# INFLUENCE OF COLD

# ON HOST-PARASITE INTERACTIONS

PART III



Editor

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# COLD AND COLDS

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ABSTRACT

Two problems arise: (1) Does cold really precipitate colds in individuals? (2) Why are colds commoner in summer? Are these two problems or only one problem? (1) The popular belief that cold or damp can precipitate a cold is very strong. Experiments at the Salisbury Common Cold Unit failed to confirm the notion. Can the belief be due to popular confusion between cause (fall in temperature) and the earliest symptom (a feeling of chilliness); or does this effect of cold only act in certain infrequent individuals or at certain infrequent moments, when virus in a latent state can be activated? Chilling does not seem to activate colds in small isolated communities in the Arctic or elsewhere. (2) Colds are certainly commoner in temperate zones in winter than in summer. Many observers report three waves - one in September-October, one in January, and often a third, lesser one in March. Very many things can be correlated with change in season; but which are the important ones? There is little evidence that temperature is itself the direct cause. Rather better, but still unconvincing, evidence might implicate humidity, perhaps because cold viruses could survive better in the air at low relative humidities. A "winter factor" could operate in such a way, favouring the virus' survival, or indirectly through change in people's habits, again permitting more cross-infections to occur. There may be "conditioned epidemics", a virus such as Influenza being seeded into a population but not manifesting itself until conditions, perhaps meteorological ones, are favourable. Possibly the physiology of the respiratory mucosa is all important. A homoeostatic mechanism may not respond promptly enough to environmental changes and virus attack may be favoured at this "unguarded hour". Recent work at Salisbury and elsewhere, permitting identification and titration of "Rhinoviruses" offers hope of a direct attack on some of these questions. Understanding of the natural history of colds may offer better hope of controlling such infections than a programme directed towards specific viruses.

I have not come all the way to Alaska to tell you what the relation between cold and colds is; rather, I have come in hope that in the discussion following my remarks some light may be shed on the obscurity which veils this subject. Why are colds called "colds"? Is it because the subject feels cold, or is it because chilling is thought to precipitate an attack? Or perhaps because colds come at the cold season of the year? We have really to consider two prob-

lems which may or may not turn out to be one and the same problem: 1) Does chilling really set off a cold infection and if so how? 2) Why are colds commoner in winter than in summer? In other words, is the seasonal prevalence of colds simply due to a summation of the effect of chilling on individuals? At the moment there is good scientific evidence that colds are in fact commoner in winter. Evidence as to the effect on the individual is more doubtful.

## Cold and Colds in Individuals

Many people are quite confident that chilling will bring on a cold in them, and they nearly all say that this can happen within an hour or two. A friend of mine has to take time off when she wants her hair washed, as it must be dried immediately and properly, otherwise she "invariably gets a cold". We have offered to test this experimentally but have met with no co-operation.

Experiments were conducted at the Common Cold Research Unit at Salisbury to test the effects of chilling. In this unit, volunteers come for ten days at a time, having offered their services as "human guinea-pigs". They are kept isolated, usually in pairs, and appropriate precautions are taken so that there is no subjective bias in deciding whether or not our experimental procedures have given them a cold. In an experiment several years ago, we took three groups of six volunteers (Andrewes, 1950). The three pairs in one group were given diluted virus, which was expected to produce only a few colds. Another six were soaked in hot baths and then made to stand, undried, in their bathing dresses in a draughty corridor for 30 minutes. After that, they wore wet socks for some hours. A third group of six received the dilute virus plus the chilling treatment. Chilling alone produced no colds. The "Virus alone" group got two colds, while the group with virus plus chilling developed four colds. Interesting, but not statistically significant. We repeated the experiment and again chilling alone did nothing. But in the other two groups the result was reversed; there were fewer colds with chilling than without it. In a third experiment our volunteers went for a walk in the rain; on returning rather cold and tired, they found that we had turned off the heat in their quarters. They were treated in three groups as before, and, as in the second test, chilling was not seen to

have any effect in predisposing to colds. Dowling et al. (1958) reported similar findings.

There is quite an extensive literature, reviewed by Thomson and Thomson (1932), on the effect on the nasal mucosa of chilling applied locally to the nose, to the whole body, or to the feet. The experiments have mostly been carried out in hope of determining why chilling causes colds. No evidence is adduced on the preliminary question of whether chilling causes colds. Mudd and Grant (1919), amongst others, found that cooling the body surface causes blanching of the upper respiratory mucosa, and a fall in temperature there of rather less than $1^{\circ}$ C. Cooling of the feet alone was rather less effective. Different observers are not in agreement as to whether local draughts playing upon the nose are more or less effective than cooling of the body generally. Schmidt and Kairies (1931) confirmed other observers that chilling caused mucosal ischaemia. This happened to everyone they tested, but the rate of return to normal was very variable, a fact having possible bearing on varying susceptibility to colds. There is one report that chilling causes increased acidity in the saliva with a suggestion that nasal secretion may be similarly affected; this, if true, could perhaps be related to the rather acid conditions which common cold viruses seem to like in tissue-culture. Draughts could, of course, operate by causing local desiccation and hence temporary stagnation in the sheet of mucus which is normally flowing continuously backwards over the mucous membranes... But I am in danger of becoming fascinated by these reports of thirty years ago and am being entrapped into discussing "how" and avoiding the question of "whether".

I offer for discussion three possible explanations of the conflict between popular belief in this matter and our own experimental findings.

The first is that the popular belief is a fairy-tale, having no real basis in fact. The second is that people confuse early symptoms with cause. Assuming, as I do, that a common cold is essentially a virus infection, it is hard to explain, on any hypothesis, how this could be "full-blown" within an hour or two of the chilling episode, as is usually reported. Is it not more likely that an early symptom of the virus infection is an undue sensitivity to the effects of the

physiological adjustments which follow a fall in temperature? Most people not infrequently get wet feet or sit in draughts without feeling chilly and without a subsequent cold; these incidents they forget.

I must digress before putting forward my third explanation. There is evidence as regards several respiratory virus infections, and more particularly influenza, that virus may be widely seeded into a population and yet not give rise to an immediate epidemic. Something has to be right before the outbreak can get started. Particularly remarkable was the way in which the A2, commonly called Asian influenza, spread rapidly in tropical countries, yet was seeded fairly freely into North America and Europe some months before anything very much happened there. With onset of cooler weather, the epidemic broke out. Common cold viruses have occasionally been isolated from normal noses and it is not unlikely that in some people they, like some other viruses, may be in a state of unstable equilibrium with their host, awaiting activation by an appropriate stimulus. We may not have induced in any of our comparatively few volunteers the right kind of equilibrium with the virus we gave them; it may be only a small percentage of the population in which chilling would upset a balance and unleash a cold.

One thing seems fairly certain. In small isolated communities in the Arctic, Antarctic, or elsewhere, cold viruses are lost or else the members of the small community soon become immune to the viruses circulating amongst them (Paul and Freese, 1933). Chilling does not induce colds in them unless there is some contact with the outside world which could possibly introduce fresh viruses. Many of you will be able to tell me if I am misinformed in this matter.

## Season and Outbreaks of Colds

Let us now turn to cold and its effects on the incidence of colds in large populations. Every chart I have seen tells the same story: colds are much more frequent in winter than in summer. One observer (Lederer, 1928) asserts that summer colds are more often sporadic and not associated with other cases in the family. In the Northern Hemisphere there is commonly a peak in the incidence of colds with the onset of cooler weather in September and October, a

second peak early in the New Year, and, less regularly, a third lower peak about March. The New Year peak corresponds to the favourite period for Influenza A epidemics. There are very many differences between summer and winter, not only in temperature and humidity, but in resulting changes in our habits, our dress, and our diet; and it is extremely difficult to pin-point any one of these as responsible for the winter increase in colds. Undoubtedly, a fall in temperature precedes an outbreak of colds, but low temperature as such does not do so; it is the change that matters. Milam and Smillie (1931) found that on the tropical island of St. John, the daily variation over a range of 6.5° C- was the same as the difference between the summer and winter maxima (6.5° C to 8° C); yet colds were virtually absent from late May to late October. They thought that with smaller temperature changes, colds were both scarcer and milder than in colder climates. Van Loghem in Holland (1928) and also observers in North America have recorded that outbreaks of colds occur simultaneously over wide areas of the country. It is very difficult to explain this on any theory of simple person-to-person spread. The outbreaks seem rather to be precipitated by temperature changes.

Several writers consider that relative humidity is more important than temperature. Hemmes et al. (1960) reported that the virus of influenza, a winter infection, survived better in the air under conditions of low than of high humidity, whereas the virus of poliomyelitis, a summer disease, behaved in an opposite manner. Hope Simpson (1958) has pointed out that with the onset of colder weather, people light fires indoors, or turn on the central heating, thus causing a considerable drop in relative humidity. At just such times he sees in his practice a sudden increase in upper respiratory infections. He does not venture to suggest whether this could be due to better survival of viruses in the air, as Hemmes' results would indicate, or to some effect on the host's resistance. The effect on virus survival seems unlikely, for rhinoviruses or common cold viruses are closely related in their fundamental properties to the enteroviruses, which include poliovirus; and their stability at various relative humidities resembles that of the poliomyelitis rather than of the influenza virus. So on Hemmes' line of reasoning, colds should be a summer disease. Further, the fall in relative humidity which Hope-Simpson records as happening in the autumn, is only

noteworthy in unoccupied rooms. In crowded rooms where cross-infection might be expected to occur, relative humidity is never very low.

What of temperature changes as causing changes in people's habits? Undoubtedly people tend to spend their spare time together indoors in the winter and much more out of doors in summer. This would tend to encourage spread of infection in winter - or so one might at first sight suppose. But if one considers workers in offices, factories, and shops (a considerable part of the population), their lives differ in summer and winter for only a small part of the day. Many of them use crowded public transport all through the year and they are perennially cheek-by-jowl in their shops, factories or offices. Probably there is better ventilation in these surroundings in the summer time, but nothing achieved by students of air hygiene has yet encouraged us to believe that respiratory infections are likely to be greatly reduced by such means; nor yet by U-V irradiation, chemical aerosols or other methods designed to give an equivalent result. The fact is that as regards colds, the experimental evidence available suggests that cross-infection takes place mainly through direct hits with infectious particles at close range rather than through minute droplet nuclei (Lovelock et al., 1952). So ventilation could hardly be expected to play a major role.

There are, of course, some circumstances in which particular kinds of habit changes leading to close aggregations certainly favour spread of virus infections. Quite a number of viruses, some types of adenoviruses, Coxsackie A 21 (or Coe virus), and Influenza B, all cause outbreaks of respiratory infections mainly in recently collected service recruits or in children re-assembling at boarding-schools after holidays. Even here season plays a role, for the adenovirus outbreaks amongst recruits are not important during summer months.

We seem to be frustrated at every turn. Every promising clue seems to peter out. Are recent advances in knowledge about colds and other viruses likely to be able to help us? I think they are. First of all, Tyrrell and his colleagues at Salisbury (Tyrrell et al., 1959) have found out how to cultivate viruses from a high proportion of common colds in adults. These we are calling Rhinoviruses (nose-

viruses). They can be grown in cultures of human embryonic kidney, and in diploid cell lines from human embryonic lung; a minority of the strains grow also in monkey kidney and other primate cells. The trick is to cultivate at a lower temperature than is conventional (33° C), at a lower pH, and in rolled tubes to give good oxygenation. New developments make their study easier. They grow in a wider range of cells; they can be studied quantitatively by counting the tiny foci of degeneration produced in cell sheets (Parsons and Tyrrell, 1961); even macroscopically visible plaques can now be produced (Porterfield, 1962). These Rhinoviruses resemble enteroviruses in being very small, ether-resistant viruses. They differ in their cultural requirements, greater lability towards acid, habitat, and pathogenicity. They are of many different serological types, and although we don't yet know how many, 30 is probably a low estimate. We are now passing the preliminary stage of establishing that these viruses do, in fact, cause many colds all over the world. The techniques developed can now be applied to studies of epidemiology. Quantitative studies are particularly needed. For many years in our work at Salisbury we could only detect virus by seeing whether or not material under study would produce colds when dropped up the noses of volunteers. Our subjects reacted very variously, and at best we could only infect 50 per cent of them. Quantitative studies were almost impossible. Now that one can count rhinovirus plaques, things are very different. It should not be difficult to discover the whereabouts of virus in the environment, and just when, how, and in what quantity it is shed from an infected person. I should not be wholly surprised to discover that virus shed by a cold-sufferer in summer was quantitatively less than in winter, so that there was less danger of infecting others. All sorts of other quantitative studies should be applicable from now on, including those concerned with seasonal variation in resistance to colds.

## Resistance to Colds

Jackson and Dowling (1959) in Chicago produced evidence that resistance to five strains of colds was specific, directed against a particular strain of virus. Discovery of the serological multiplicity of cold viruses fits in with this. Antibodies to particular strains seem to be well correlated with resistance to those strains. Yet other

evidence suggests that there is a non-specific element in immunity. It is generally agreed that in isolated communities immunity to colds falls, so that contact with civilization after a long interval is quickly followed by colds, and often they are severe ones. Why? The usual explanation is that people in a large community are constantly subjected to little doses of virus which reinforce their immunity, often without giving rise to any symptoms. Yet if there are 150 or 57 or some other large number of cold viruses, it is unlikely that we are all being regularly exposed to all of them. If not, why are we so much more resistant than these lately-isolated people? And why don't they get 57 colds when they begin to mix with society? It seems that if there is a nonspecific as well as a specific immunity, the facts could be reasonably explained. This could be mediated by interferon, a virus-inhibiting protein produced by cells under the stimulus particularly of dead or damaged virus. Its production seems to supply a quickly acting method of halting a virus infection until antibodies can be made and mobilized. Its action is local and nonspecific, being directed not merely against the virus which evoked it. In tissue-culture, rhinoviruses are amongst those most susceptible to its action, and the Salisbury workers are at present engaged on studies in volunteers of a possible role of interferon in cold virus infections.

If I had to offer a working hypothesis as to the effect of cold in favouring colds, it would be along the following lines. The human body, especially the respiratory tract, is exposed to large environmental changes. The complicated anatomy of the nose is part of a homeostatic mechanism designed to protect the lungs from sudden changes. But it may not operate completely and instantaneously. There may be a lag, and during an "unguarded hour", a virus reaching or already in the mucosa, in equilibrium with the host, may snatch its chance. I doubt whether a local fall in temperature alone suffices; it may well be an indirect consequence of temperature fall. Some evidence suggests that interferon production is not so good at lower temperatures, and that is the sort of thing about which I am speaking.

I regard an attack on this difficult matter as the most important phase of our war against respiratory infection. Injecting vaccines against dozens of sero-types of virus seems to be a rather unpromising business. Discovery of the relation between cold and colds

could lead to measures effective not only against rhinoviruses but against all the other respiratory plagues as well. I feel that the balance may be tipped by something quite small but devilishly elusive. Perhaps our discussions at this symposium will bring forth a clue.

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## DISCUSSION

BLAIR: Sir Christopher, I was particularly interested in your viewpoint with regard to the effect of chilling. I never had occasion to do a study of this particular matter with regard to our patients whom we have cooled down to hypothermic levels, and in the last 100 patients I can recall for whom I have been re-

sponsible, there has not been one single instance of a cold, and furthermore, no pneumonia, so I think that this would support your idea that chilling is probably not of any importance in the development of a cold.

CAMPBELL: Of course, it's been a long time since I have had bacteriology; we used to think that bacteria may be involved here. You didn't mention bacteria at all. I just wondered whether in your studies you had found any correlation between bacterial flora and virus infection?

ANDREWES: Well, I think everybody agrees that in the late stages of the cold, secondary infection with bacteria comes and causes the sinusitis and the yellow. muco-purelent stuff. This is generally about in the early stages of the cold; it is very difficult to find anything abnormal about the bacterial flora then, but there is no doubt that in the later stages they do come in and confuse the picture.

CAMPBELL: But no particular type, I mean.

ANDREWES: No, it varies, I think.

WALKER: Have you done any studies on immunity to the common cold? Jackson and Dowling, I believe, report a surprisingly slow development of the immunity.

ANDREWES: Well, yes, we are studying that, and we agree with them that the immunity seems to be specific. We have one of the people working at Salisbury, Dr. Periera, who is the origin of the HGP strain of virus, and he has been bled at intervals. His serum, before he had a certain cold in 1957, had no antibodies to his strain. Then he had this attack of cold and his antibodies came up pretty quickly. Since then, he has been followed along and he has had several colds and we have gotten different viruses from him. The original HGP virus is one of the ones that grew in monkey tissue. One of the other strains was one that didn't grow in monkey, but would grow in human tissues and another was a strain which would transmit colds to volunteers, but we couldn't grow it at all. All this time he has

had only slightly falling antibody to his original strain and this has been quite unaffected by his attacks of these other colds.

WALKER: And his antibody came up in a matter of a couple of weeks?

ANDREWES: I can't recall, but fairly soon.

WALKER: As I recall, the reports of Jackson and Dowling show that this was much slower in their experience.

ANDREWES: I don't remember their doing it with antibodies.

WALKER: It seems to me they got a peak only after six months or a very prolonged period of time, which is strange.

SCHMIDT: I wonder if you might have a comment on the work that I think has been done by Kruger and others[1] concerning the concentration of the negative or positive charged ions in the atmosphere and its effect on the mucosal lining.

ANDREWES: I am afraid I don't know about that.

TUNEVALL: There is one thing that relates to that season of the year, and that is when our children return from their holiday to school. Couldn't the incidence of colds perhaps be correlated to these instances when the children get together?

ANDREWES: Well, there is no doubt that children are very much more effective spreaders of infection than adults. We carried out some epidemiological studies in a rural valley near Salisbury, and it was found that colds in adults in families in which there were school-age children were two and a half times as common as in families when there were no school-age children. There is no doubt that the little darlings spread the virus very efficiently.

1 Krueger et al. 1959. Proc. Soc. Exp. Biol. Med. 102: 355-357.

SULKIN: You mentioned that to limit the occurrence of the common cold through classical immunization might present a difficult problem because of the growing number of serologic types. I wonder, in your opinion, if it is conceivable that a new type of immunization procedure might be evolved through the mechanism of interference. That is to say, since inactive viral particles will produce interferon, is it conceivable that by introduction of say, influenza virus into the external nares, that one might manufacture sufficient interferon to cope with any one of these common cold viruses?

ANDREWES: We have actually tried that and it didn't come off, but we don't despair of an approach on that line. Issacs found that the production of interferon is not only mediated by virus approach; he thinks it is fundamentally a reaction to any foreign nucleo-protein approach, and it is possible to produce some interferon with nucleo-protein approaches of non-viral origin. One man made an interesting suggestion which we haven't followed up; that is, that the low incidence of colds in the summer might be due to the fact that in the summer, people are constantly being stimulated with nucleo-protein produced from pollen.

NUNGESTER: I hate to agree with Dr. Blair and sell Joe Berry's long underwear short here, and I wonder if you believe that experiments with humans where there is more or less uniform cooling are characteristically comparable to the sort of things that you have done at Salisbury. In keeping with the work of Mudgrant and Goldman,[2] it isn't the uniform cooling of the whole body that is important, but the irregular cooling of parts of the body that is important.

WALKER: And I would add, to the right temperature.

NUNGESTER: Yes.

ANDREWES: Our feeling about this effect of cold is that the

2 Annals of Otology, Rhinology, and Laryngology. 30: 1. 1921.

way we did the experiment it didn't show anything, but we are not willing to say that there isn't a relation. We failed to achieve it, and we hope other people will find a way they can do it; then we will believe them. We are not biased about the whole thing.

NUNGESTER: A few years ago, we got to playing with this sort of thing. In the process of taking nasal washings, we took just a little bit of a history of the people from whom we took the nasal washings, if they had a cold. These are students, of course; they like to brag on how hard they have been working, so you have to discount this a bit, but it seems that there was almost a pretty good correlation between loss of sleep and the incidence of a common cold. There seems to be more of a correlation with this sort of fatigue and stress than there was with exposure to cold. Have you had any experience with fatigue? Of course, you have told us that you brought the fatigue element in.

ANDREWES: Yes, also the opposite effect; if people go away for a summer holiday and come back feeling absolutely on top of the world, they're likely to get a cold almost at once.

SCHMIDT: In connection with humidity and its possible effect, it occurred to us, during our studies here in Alaska, that it might be involved. Humidity is very low during the winter months in Central Alaska, to the extent that you waken with a very dry and crusty throat. It is hard to imagine that one wouldn't be most susceptible to any sort of respiratory illness under these conditions. When Dr. Beard, of the Armed Forces Epidemiological Board (at that time, at least) was here, I discussed this aspect with him and he indicated that he had considered this to be a factor some years ago. He investigated this by working in a desert area where it was warm but very dry, and he didn't observe a higher incidence of infection in his subjects. Although our attempts were rather clumsy and humble, no relationship could be established between the relative humidity and upper respiratory infection. Even though you are extremely uncomfortable, you seem to survive very nicely.

ANDREWES: Of course, under those desert conditions is the

time when you get epidemics of cerebral spinal fever. I think that seems to go in the Sudan and other regions around the Sahara.

CAMPBELL: I would like to ask a fundamental question. It has nothing to do with cold exposure, but in immunization to viruses that seem like polio, you have to have the specific strain to produce immunity. We are so conditioned with the pneumococcus work in which the polysaccharide plays a major role in infection. How do you envision the structure of a virus that is so specific? There must be something in common with all these strains of cold viruses, and if we had enough, you probably could show immunologically or serologically that there were cross reactions, like the C substance in pneumococcus. Do you envision a capsule or something around the virus?

ANDREWES: Most of these viruses consist of nucleo-protein core and the other protein outside, and in the case of the smaller viruses, that is about all there is to it. We have found evidence of a very slight amount of cross-reaction between some of these colds. It is only very trivial, but there are small amounts, as in the case of the pox group of viruses covering not only all the animal poxes, but also myxoma and a number of others. It has quite recently been shown by Japanese workers, and by Fenner in Australia, that there is a common nucleo-protein antigen which was overlooked for many years. I wouldn't at all think it impossible that a similar thing would be found with some of these smaller viruses. Whether it would be of any use in inducing immunity would be anybody's guess.

WALKER: Most commonly, this nucleo-protein represents the common antigen of the group. It is not a protective antibody.

CAMPBELL: Well, you could modify it some way. They must have something in common. They like to live in the nose; diphtheria likes to live a little further down, but both bacteria and viruses, of course, are local; that's where you get the term neurotropic and dermatropic.

ANDREWES: We need to collaborate with an immunochemist.

CAMPBELL: Next year, I will be over.

NORTHEY: I'd like to go one step further. How do you feel that antibody really acts in these infections?

ANDREWES: Well, it seems to play more of a part than we expected. With some of these other respiratory infections, you seem to be able to get repeated infections in spite of the presence of some antibody in the serum, but in the case of the common cold viruses, it appears that if you have got an antibody of that particular strain, you are likely to resist challenge by that particular strain. We were really surprised to find that there did seem to be considerable relation between antibody and immunity.

METCALF: Is there a difference between the antibody titer that you find in the serum and the nasal secretions, and if so, is this likely to be of any significance?

ANDREWES: Well, we have looked for neutralizing things in the nasal secretion, but that was some time ago before we really got on to the way to grow these viruses, so I don't think we know the answer to that question. In the case of influenza, of course, you do get the same kind of antibody in the nasal secretions as you do in the serum, but much less of it.

METCALF: I was thinking of the obvious relationship of a fluid bathing a vulnerable point of cells subject to attack.

ANDREWES: Of course, all the earlier work in that is muddled up by the fact that people didn't appreciate the possible presence of interferon.

PREVITE: Just one naive question, not being very familiar with virology. We often hear that there are many different viruses capable of causing what we call the "common" cold. Has anyone made any effort to ascertain how great and variable are the number of viruses in any given area?

ANDREWES: There have been a number of studies of that. Dr. Hamre in Chicago has published some work on that, and it

appears that it depends on what age group you take. If you take small children, you will find that the rare influenza viruses produce quite a lot of infection. In any age group, the respiratory syncytial virus may be prevalent in one year, and in another year it may be completely absent. Rhinoviruses seem to be the hard core which cause more colds than anything else, but I should make it clear that there are still a great many colds from which we havn't been able to cultivate any viruses, although these things will still produce colds in volunteers. So there is still quite a lot to learn, but I think the proportion of the colds caused by different agents probably varies very much from time to time and from place to place.

Well, I don't know that anybody has actually solved all my more difficult problems for me, but anyway, I am very grateful for all the suggestions that have been made in the matter.

# EFFECT OF ENVIRONMENTAL TEMPERATURE ON VIRAL INFECTION[1]

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ABSTRACT

In considering the effects of cold on viral infections, four questions seem to be of particular importance. These are: (1) Can exposure to cold cause an acute but mild and inapparent infection to become an apparent and severe disease? (2) Can it seriously worsen an apparent viral infection? (3) Can it activate a latent viral infection? (4) What are the mechanisms by which cold exerts an effect on viral infections? Indication that the answers to the first three questions can be "yes" is available from studies of infections in animals. Studies in this laboratory on Coxsackie infections in mice are pertinent to the first question. In infant mice the Conn.-5 strain of Coxsackie B-1 virus causes a generalized, lethal infection, but in adult mice the infection is limited to a mild, inapparent pancreatitis. Exposure of adult mice to a 4° C environment, however, results in illness with essentially 100 per cent mortality. Pertinent to the second question are studies on the myxoma-fibroma viruses. Marshall has shown that exposure to cold increases the severity of disease in rabbits infected with attenuated strains of myxoma virus. Relative to the third question, Shope has found that exposure to cold weather appears to activate latent infections of swine influenza virus in swine. Although the mechanisms by which cold exerts an effect on viral infections have not been studied extensively, there is growing evidence that it may be by simply lowering tissue temperature to one more favorable for multiplication of the infecting virus. At normal temperatures Conn.-5 Coxsackie virus multiplies only in the pancreas of the adult mouse. Exposure to 4° C results in reduction of body temperature by 1.0° C to 1.5° C and in multiplication of virus in many organs. Exposure of mice to 36° C raises their body temperature 2° C and inhibits multiplication in all tissues, including the pancreas. Study of Conn. -5 virus multiplication in in vitro cultures reveals that the virus does not multiply well in adult mouse tissues at 37° C or at higher temperatures, but does multiply well at 35° C.

1 Studies by the author on this subject were supported by grants from the National Institute of Allergy and Infectious Diseases and the National Cancer Institute.

Studies concerned with the effect of environmental temperature on viral infections have sometimes been confusing and seemingly contradictory, but cold has most often been found to aggravate viral infections. For the purposes of this symposium, then, it seems to me that there are four questions that should be considered: 1) Can exposure to cold cause a mild, inapparent viral disease? 2) Can this exposure seriously worsen an apparent viral infection? 3) Can it activate a latent viral infection? 4) What are the mechanisms by which cold exerts its effect on viral infections?

It is difficult to find direct experimental data bearing on these questions that have been obtained in studies of infections in man, but some evidence is available from studies in animals, and some information is available from study of viral infection of cells in culture. The data are not extensive, but I think they provide indication that the answers to the first three questions can be yes, at least with selected virus-host systems and under laboratory conditions, perhaps even in man, and that we may find such effects if we make the proper search.

I should like, then, to disclose this evidence. Some of it is from others, but I shall limit consideration to that which I think has a quite direct bearing on the questions that I have posed.

## EXAMPLES OF THE EFFECT OF COLD ON VIRAL INFECTION

### Can Exposure to Cold Cause a Mild, Inapparent Viral Infection to Become an Apparent and Serious Disease?

Boring and I have studied a model viral infection in mice caused by the Conn.-5 strain of type B1 Coxsackie virus (Boring, ZuRhein, and Walker, 1956; Walker and Boring, 1958). Although this virus produces a generalized and lethal infection in infant mice, it causes in adult Swiss mice only a pancreatitis. The mice seldom show outward signs of illness even after very large inocula. However, when

| Inoculum (i. p.) | Environmental temperature | Deaths**/no. inoc. |
|---|---|---|
| Virus Suspension* | 25° C | 0/20 |
| " | 4° C | 19/20 |
| Normal mouse tissue suspension | 25° C | 0/20 |
| " | 4° C | 0/20 |

Figure 1. Lethal effect of Coxsackie virus infection in adult mice at 4° C. *4000 $LD_{50}$ for infant mice. **No. of mice dying during 10 days of observation.

| Ambient temperature C | | |
|---|---|---|
| Before inoculation* | After inoculation | Deaths/no. inoculated |
| 4° for 2 days | 25° | 0/12** |
| 25° | 4°, 1 day, then 25° | 0/12 |
| 25° | 4°, 2 days, then 25° | 0/12 |
| 25° | 4° | 12/12 |
| 25° | 25°, 1 day, then 4° | 12/12 |
| 25° | 25°, 2 days, then 4° | 9/12 |
| 25° | 25°, 4 days, then 4° | 9/12 |
| 25° | 25°, 6 days, then 4° | 0/12 |

Figure 2. Relationship of time of exposure to cold and lethal effect of Coxsackie virus in adult mice. *1500 infant $LD_{50}$ given i. p. **Deaths in 10 day observation period after mice placed at 4° C.

inoculated mice are placed in a room at a temperature of 4° C, the infection then becomes quite uniformly lethal. Figure 1 shows a typical experiment. Acute, limited exposure to cold is not sufficient to change this infection in adult mice from a restricted, asymptomatic one into a fatal infection. Continued exposure through several days is necessary (Fig. 2).

That the deaths of inoculated mice at 4° C are related to viral infection can be shown by neutralizing the virus with specific antiserum prior to injection or by passively immunizing the mice prior to inoculation. This prevents the deaths at 4° C. In addition, it can be shown that this phenomenon is not caused simply by inability of mice with pancreatitis to survive in the cold, but is due to a real enhancement of the infection. Measurement of virus levels and study of tissue histology indicate that at ordinary temperatures viral multiplication is limited to the pancreas, but that in mice at 4° C, viral multiplication and tissue damage takes place in many tissues. Data on this point will be presented in a later section.

An investigation of Briody and associates (Briody et al., 1953) concerned with what could well be called inapparent infection is also pertinent here. These workers examined the effect of cold on the process of adaptation of influenza A' virus to multiplication in the lungs of mice. Unadapted influenza virus usually multiplies to some extent in mouse lungs, but it causes little pneumonia or mortality until after a series of serial passages has resulted in a selection of virus capable of rapid and abundant multiplication in the lungs of mice. When inoculated mice were maintained at 5° C, however, the virus grew to 100-fold higher levels, the extent of pneumonia was increased and mice began to die of influenzal pneumonia after only a few passages.

## Can Exposure to Cold Seriously Worsen an Apparent Viral Infection?

This question, of course, is not very different from the previous one, because in many instances the difference between inapparent and apparent infection is only one of degree. Nevertheless, the question serves well to introduce work on myxoma virus infections.

## TEMPERATURE AND VIRAL INFECTION

The effect of environmental temperature on viral infection has appeared to be of some practical importance in Australia in the evolution of myxomatosis in wild rabbits. Marshall (1959) noted that there was repeated suggestion that myxomatosis spreading naturally through wild rabbits was more lethal in winter than in summer, and Mykytowycz (1956) observed that rabbits experimentally infected with an attenuated strain and housed in unheated quarters had a higher mortality rate in winter than in summer. To test the possibility that these observations were related to ambient temperature, Marshall exposed inoculated rabbits to fluctuating cold (-1° C to +1° C for 16 hours and 15° C for 8 hours each day) and compared the results with those at normal room temperatures (20° C to 22° C) and at elevated temperatures (37° C to 39° C for 16 hours and 26° C for 8 hours each day). These fluctuating temperatures were chosen to simulate day and night fluctuations of winter and summer. He found that ambient temperature had little effect on infections with a highly virulent strain of myxoma virus or with the quite virulent rabbit pox. But if rabbits were inoculated with an attenuated strain of myxoma virus that at 22° C caused death of about 60 per cent of rabbits, then exposure to cold increased the mortality to over 90 per cent and exposure to heat reduced mortality to about 30 per cent (Fig. 3). Myxomatosis in a rabbit is a very distinctive disease, and comparison of the signs of disease in the rabbits at the various temperatures, as well as differences in the levels of virus in the blood, were fairly convincing indications that the differences in mortality rate were due to alterations of the extent and severity of infection instead of to some other effect of the temperatures.

Somewhat similarly, Sulkin (1945) has shown that after inoculation with influenza A virus, mice maintained at 15.5° C have significantly more pulmonary consolidation and mice at 35° C less consolidation than do mice held at 21° C to 25° C. And I judge from the abstracts of Drs. Metcalf and Marcus that they will be providing additional data pertinent to this question.

### Can Exposure to Cold Activate a Latent Infection?

Latent here means an inapparent infection that exhibits chronicity and some degree of host-virus equilibrium. Although this is a par-

| | Ambient temperature | | |
|---|---|---|---|
| | -1° C to +1° C for 16 hrs; 15° C for 8 hrs per day | 20° C-22° C | 37° C to 39° C for 16 hrs; 26° C for 8 hrs per day |
| Infected rabbits | 36/39 (92%) | 22/35 (63%) | 9/30 (30%) |
| Uninoculated control rabbits | 0/36 | | 0/31 |

Figure 3. Mortality in rabbits infected with an attenuated strain of myxoma virus and held at different temperatures. (Marshall, 1959).

ticularly interesting question, there seems to be relatively little data that concerns such infections and involves hosts and viruses that lend themselves to detailed study. Dr. Andrewes has already dealt with the common cold in man. There is one study, however, that should be mentioned here.

Shope has pointed out that circumstantial evidence concerned with epizootics of swine influenza has long indicated that the stimulus responsible for precipitating attacks of the disease in swine latently infected with the virus is in some way associated with sudden changes in weather and especially with the onset of cold, wet weather. He performed an experiment (Shope, 1955) in which he prepared 25 swine by feeding them earthworms containing lungworm larvae carrying swine influenza virus. After about 30 days, during which the animals remained well, he then exposed them for from 4 to 24 hours to adverse weather conditions, which always included rain or snow and low temperatures. No data were obtained on the effect of this on the body temperatures of the animals. Eight uninoculated controls and 15 inoculated animals remained well, but 4 swine did develop the typical illness of swine influenza and 6 others developed serological evidence of infection.

## POSSIBLE MECHANISMS FOR THE EFFECT OF COLD ON VIRAL INFECTIONS

The studies that I have outlined indicate that with some hosts and viruses under proper circumstances cold can aggravate and activate viral infections. I have limited discussion here to those studies that do point to these possibilities, because I think failures to find an aggravating effect of cold, or even the occasional report of the opposite effect, are only to be expected with some host-virus combinations and under certain circumstances. But the fact that there are a good many reports of no effect or a protective effect of cold must be kept in mind in any evaluation of possible mechanisms. I shall discuss this further in a later section.

In any consideration of mechanisms that might account for the effects of cold on viral infection two possibilities quickly come to mind. One is that host defenses are modified by exposure to cold, and the second is that cold acts as a stressing agent and modifies the host through alteration of hormone balance.

The host defense most frequently considered and seems particularly pertinent in viral infection is antibody production. The effect of cold on antibody production has been discussed by others in this meeting, but I want to point out that Marshall (1959) considered the possibility of inhibition of antibody production in his study of rabbit myxomatosis and showed that under the conditions of his experiments antibody against sheep erythrocytes developed as rapidly and to as high levels in rabbits exposed to cold as in control rabbits.

In our study of Coxsackie virus infections in mice, Boring and I have never actually measured the antibody developed against Coxsackie virus in mice at 4° C, because we found that cold still exerted its effect even if we delayed exposure of infected mice until the antibody producing process was well under way (Boring et al., 1956). Specific neutralizing antibody appears in the blood of mice on the third day after inoculation with small quantities of Coxsackie B1 virus and on the fourth day the antibody is at substantial levels (Fig. 4; Boring and Walker, unpublished data). Even though exposure to cold is delayed until the fourth day, its effect is not nullified (Fig. 2). This suggests to us that even if cold were found to have an inhibiting effect on antibody production, this would still not explain the influence of cold on the infection. In addition to this, current work using mice thymectomized at birth indicates that an adult mouse does not develop a generalized lethal infection with Coxsackie B1 virus even though the mouse is incapable of producing antibody.

Other host defenses such as non-specific viral inhibitors and interferon may conceivably be altered by cold, but there is little positive evidence for this, as yet.

The role of stress is difficult to assess. There is little question that the cold exposure used in the studies that I have discussed were of a degree sufficient to cause some of the physiological changes identified with stress. And treatment of animals with large doses of

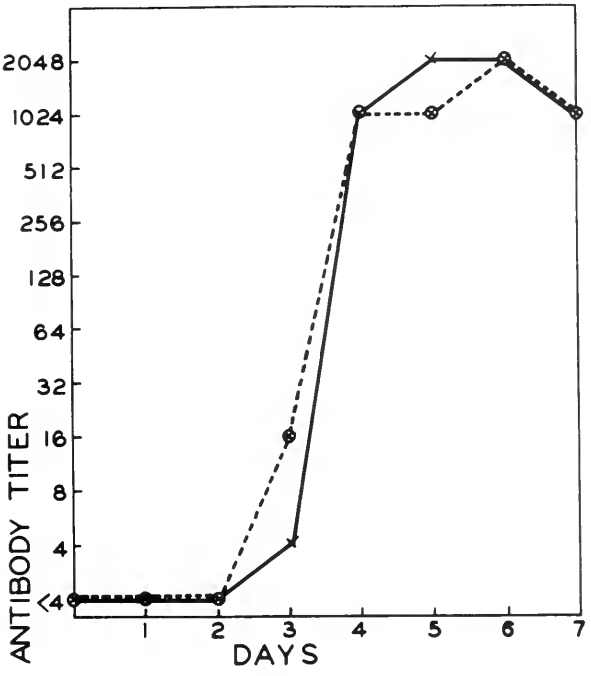



Figure 4. Antibody response to Coxsackie virus infection in mice at 25° C ambient temperature. Adult Swiss mice were given 280 infant mouse $LD_{50}$ i. p. The antibody titers from two experiments are plotted as separate curves. Antibody assays were made in infant mice and antibody titers are expressed as the reciprocal of the serum dilution that neutralized $LD_{50}$ of virus.

cortisone causes an aggravation of a number of viral infections similar to that seen with exposure to cold. Administration of 2.5 mg of cortisone to adult mice infected with Coxsackie B1 virus results in a generalized and lethal infection quite similar to that produced by exposure to cold (Boring, Angevine, and Walker, 1955). But Boring and I (1958) were not able to produce similar effects by treating mice with physiologically active doses of ACTH. In addition to this, the fact that short exposures to cold were not effective, that adaptation of mice to cold did not nullify the effect, and that exposure to the stress of heat caused an entirely different response has led us to think that just the stressing effect of cold cannot account for its effect on viral infection.

I have indicated some skepticism that inhibition of antibody pro-

| Tissue | Day of infection | Virus titers 4° C | 25° C | 36° C |
|---|---|---|---|---|
| Blood | 2 | $10^{-4.3}$ | $10^{-4.6}$ | $< 10^{-1.0}$ |
| | 4 | $10^{-3.7}$ | $< 10^{-1.0}$ | $< 10^{-1.0}$ |
| Brain | 2 | $10^{-1.5}$ | $10^{-1.5}$ | $< 10^{-1.0}$ |
| | 4 | $10^{-2.1}$ | $< 10^{-1.0}$ | $< 10^{-1.0}$ |
| Pancreas | 2 | $10^{-6.9}$ | $10^{-7.7}$ | $< 10^{-1.0}$ |
| | 4 | $10^{-6.7}$ | $10^{-5.7}$ | $< 10^{-1.0}$ |
| Heart | 2 | $10^{-3.75}$ | $10^{-3.5}$ | $< 10^{-1.0}$ |
| | 4 | $10^{-4.4}$ | $< 10^{-1.0}$ | $< 10^{-1.0}$ |
| Liver | 2 | $10^{-4.5}$ | $10^{-4.0}$ | $< 10^{-1.0}$ |
| | 4 | $10^{-5.0}$ | $< 10^{-1.0}$ | $< 10^{-1.0}$ |

Figure 5. Virus in tissues of adult mice infected with Coxsackie virus* and held at 4° C, 25° C, or 36° C. *Inoculated with 140 infant mouse $LD_{50}$ i. p.

duction or stress can account for the effect of cold on viral infections. For discussion of another possible mechanism I want to return to the model of Coxsackie virus infections in mice. In studying the effect of cold on this infection, Boring and I (1958) followed the fate of virus in various tissues of infected mice. It was evident that in mice at normal temperatures an initial viremia was followed by significant viral multiplication only in the pancreas. But in mice at 4° C, the virus multiplied to relatively high levels and produced marked damage in several tissues. It was also found that if mice were maintained at an elevated ambient temperature, viral multiplication was inhibited in all tissues, including the pancreas (Fig. 5). In fact, even if the pancreatitis was allowed to progress for 36 hours after inoculation, exposure to 36° C still brought about a prompt drop in virus titer in the pancreas and rapid elimination of the virus. An important point is that when rectal temperatures of mice are measured during exposure to such temperatures, it can be

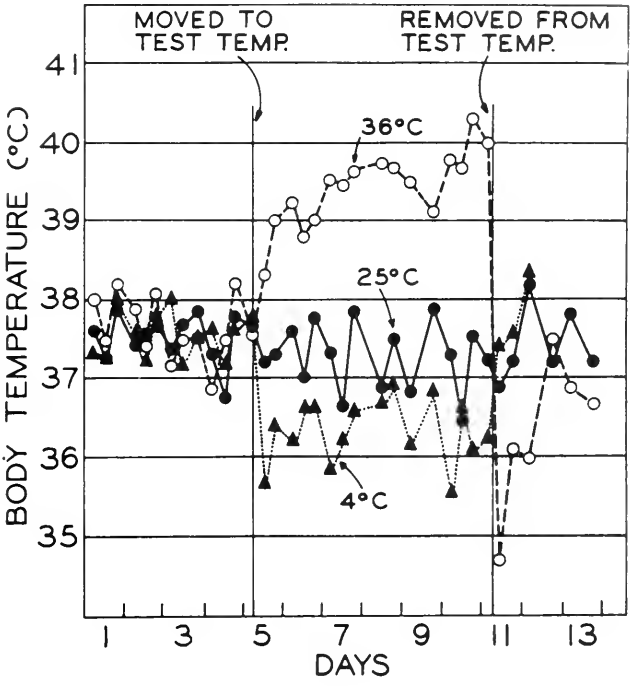



Figure 6. Effect of environmental temperature on the body temperature of mice. Each point represents the mean of the rectal temperatures of 10 mice. (Walker, D. L., and Boring, W. D. 1958. J. Immunol. 80: 39-44.)

demonstrated that at 4° C the mouse's internal temperature is lowered about 1° C to 2° C and exposure to 36° C raises the rectal temperature about 2° C to 3° C (Fig. 6).

These phenomena suggest that in the host cell there are temperature-sensitive reactions that can control Coxsackie virus multiplication. Additional support for this has been provided by Boring and Levy (1962) who demonstrated that in HeLa cells in vitro the optimum temperature for multiplication of the Conn.-5 strain of Coxsackie virus is 36° C and that multiplication is markedly inhibited at 38° C to 39° C, and is also reduced at temperatures below

| Temperature of incubation | Virus | |
|---|---|---|
| | Fibroma | Myxoma |
| 29° C | 5.5* | 5.5 |
| 33° C | 7.5 | 6.5 |
| 35° C | 7.5 | 7.5 |
| 37° C | 6.5 | 7.5 |
| 40° C | 4.5 | 7.5 |

Figure 7. Multiplication of myxoma and fibroma viruses in primary rabbit kidney cells at various temperatures. *Log rabbit skin infectious units per ml produced in 48 hours.

36° C. We have recently been looking at this more directly by comparing the multiplication of B1 Coxsackie virus in primary cultures of adult and infant mouse tissues at various temperatures in order to determine the optimum temperature for multiplication in the tissues usually affected in the mouse. Our preliminary results suggest that the virus can multiply in adult tissues if the temperature of incubation is reduced to 35° C and that it is inhibited at higher temperatures, whereas in infant tissues it multiplies to high titer at 37° C to 38° C as well as at lower temperatures. Our experiments have not proceeded far enough, however, to provide really reliable data.

I have already indicated that in his study of myxoma virus infections in rabbits, Marshall (1959) found a pattern similar to the one I have described for Coxsackie virus; that is, an ameliorating effect of high temperature as well as enhancement of the infection by cold. Similar protective effects of elevated ambient temperature had previously been reported by Thompson (1938). Both Thompson and Marshall found that rabbits at test temperatures had shifts of their rectal temperatures of only about 0.5° C downward in the cold and and 0.5° C to 1° C upward in the hot room, but their skin temperatures changed 4° C to 5° C. The possibility that these temperature changes may have been important in controlling the course of the infection is supported by other evidence indicating that multiplication of myxoma and fibroma viruses is easily affected by change of temperature. The myxoma and fibroma viruses and their various vari-

ants present a spectrum of viruses that are very closely related in many physical and biological characteristics, including antigenic makeup. However, after intradermal inoculation virulent myxoma virus invades, causes generalized disease, and is almost 100 per cent lethal for domestic rabbits, while fibroma virus causes only local benign tumors that eventually regress without any apparent harm to the rabbit. It is noteworthy that the only tissues in which fibroma will multiply and cause lesions in the adult rabbit, even if injected intraperitoneally or intravenously, are the surface tissues of skin and testes. It can be demonstrated quite easily that myxoma and fibroma viruses differ markedly in their capacity to multiply at temperatures above 35° C. Thompson (1938) demonstrated this in vivo when he raised the skin temperature of rabbits by exposure to heat and showed that fibroma lesions were quite easily inhibited while the disease caused by virulent myxoma virus required higher temperatures to bring about amelioration. This can be shown in primary cultures of rabbit tissues in vitro. Kilham (1959) has provided some data on this, and some of our own data are shown in Figure 7. Fully virulent myxoma virus multiplies to high titer even at temperatures of 40° C to 41° C, while fibroma virus reaches peak titers at 32° C to 35° C and is inhibited to some extent at temperatures as low as 36° C to 37° C and is severly inhibited at higher temperatures. Variants of myxoma virus exist that are reduced in their virulence and are intermediate between myxoma and fibroma viruses in the characteristics of the disease that they produce in rabbits. It was one of these that Marshall used in his study. We are currently comparing the capacity of myxoma virus strains to multiply at various temperatures and their relative invasiveness and virulence in rabbits. This work has not progressed far, but it appears that for many attenuated strains reduction in virulence is accompanied by decreased capacity to multiply at temperatures comparable to the internal temperature of the rabbit.

Other studies indicating a relationship between virulence and capacity to multiply at temperatures above 37° C can be cited. Bedson and Dumbell (1961) have shown this relationship with several poxviruses and their virulence for chicken embryos. Most detailed, however, have been the extensive studies of Lwoff and associates on poliovirus (Lwoff, 1959; Lwoff and Lwoff, 1960, 1961). Lwoff has demonstrated in cells in culture that poliovirus is able to multiply

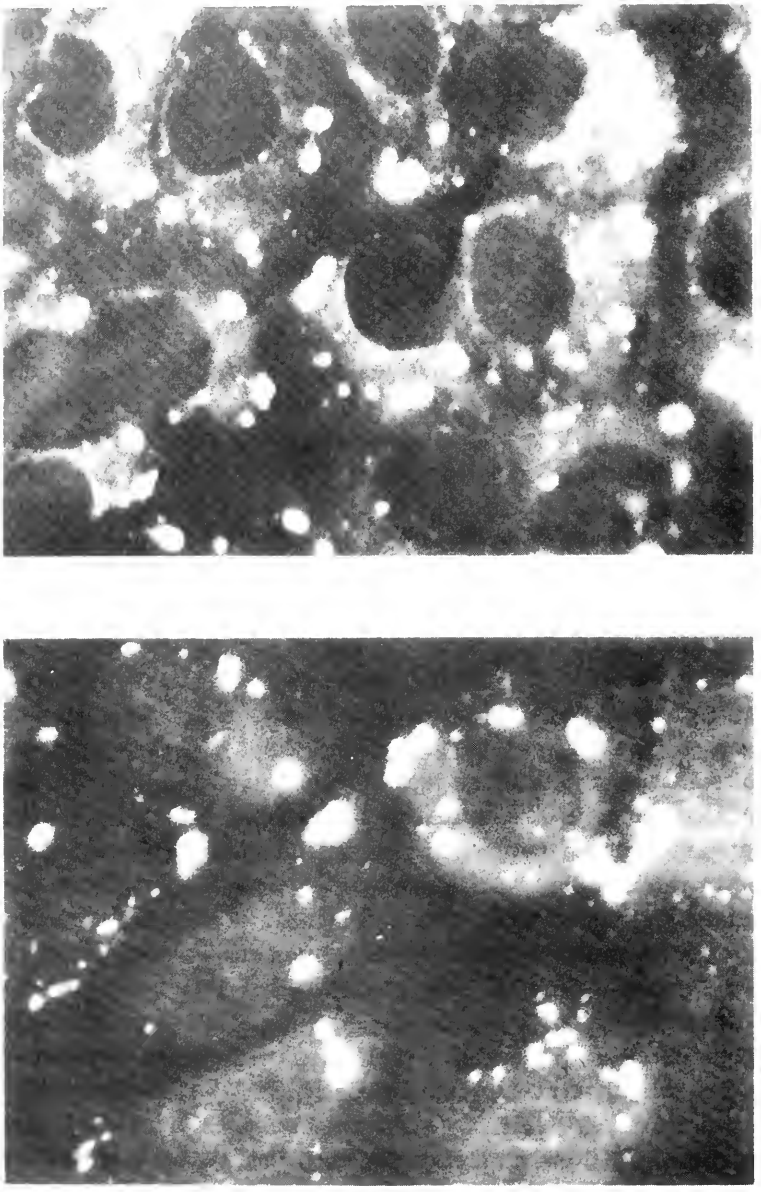
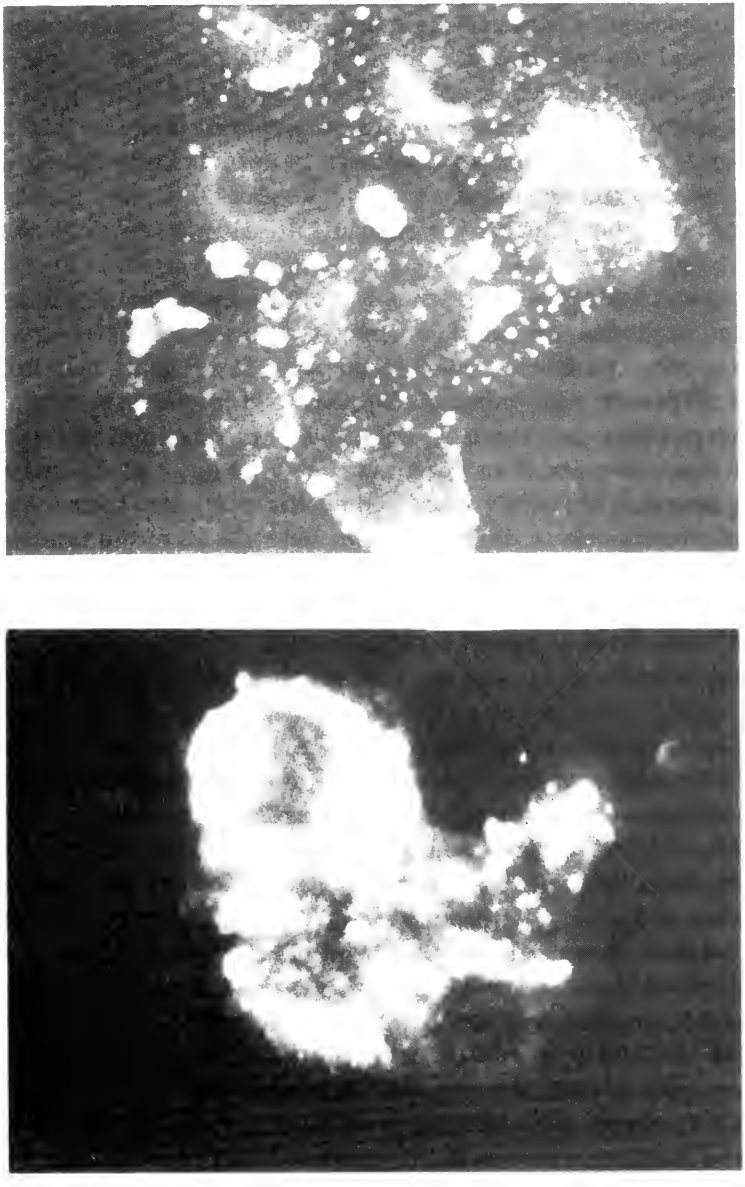


Figure 8. Carrier cultures of mumps virus in human conjunctiva cells (C-M cultures) at different temperatures. Cultures were grown at 37° C and then moved to test tempera-



tures for 24 hours. Cells fixed and stained with fluorescein-conjugated anti-mumps serum. A. 37° C. B. 40° C. C. 35° C. D. 33° C. Magnification -X 1000.

only within certain limits of temperature. For many strains a temperature of 40° C or above inhibits multiplication and viral production is depressed below 33° C. It appears that elevated temperature produces a block in the second half of the virus multiplication cycle and low temperatures block the first part of the cycle. But he points out (also Dubes and Wenner, 1957) that strains vary in the temperature that is optimum for their multiplication and that, in general, neurovirulence in monkeys is associated with those strains that are capable of good multiplication at 40° C, and lack of virulence with those incapable of multiplication at 37° C or above.

There is additional evidence from quite a number of studies using tissue cultures or chicken embryos (Enders and Pearson, 1941; Thompson and Coates, 1942; Sharpe, 1958; Hoggan and Roizman, 1959; Wheeler and Canby, 1959) that the temperature range within which viruses can multiply vigorously is often quite limited, and that the upper limit in particular may be quite sharp and abrupt. For several of the viruses studied the optimum temperature for multiplication is a degree or two below that of the internal body temperature of some of the common mammalian hosts. I want to mention briefly some data concerned with mumps virus, because it could conceivably provide some insight into how cold could affect latent infections.

Hinze and I have been studying a carrier system of mumps virus in human conjunctiva cells. This system has been maintained for several years (Walker and Hinze, in press). The cells multiply at a rate comparable to control cultures, and show little evidence of deleterious effect even though use of fluorescent antibody demonstrates that 90 per cent or more of the cells contain viral antigen. As routine, these cultures are grown at 37° C, at which temperature about 1 of every 100 cells appears to be excreting virus as judged by the fact that erythrocytes will adsorb to the cell surface. There is a low level of virus in the medium. At 37° C practically all of the cells contain antigen, but it is restricted to a few sharply outlined, discrete, masses in the cytoplasm (Fig. 8a). At 40° C the antigen is perhaps even more restricted in its distribution (Fig. 8b). But at 35° C or 33° C the antigen becomes widely distributed in the cytoplasm in small granules (Fig. 8c and 8d) and the cells tend to become rounded and ragged in appearance. At 35° C or 33° C erythro-

cytes will adsorb to 50 per cent or more of cells and the virus concentration in the medium increases by 10 to 100-fold indicating that at the lower temperatures the equilibrium between cells and virus is upset and there is much more virus production and release in the cultures.

Lwoff (1959) has emphasized the narrow zone of optimum temperature for viral multiplication and the inhibitory effect of elevated temperature to argue that fever may be an important host defense mechanism in choking off viral infection, and he has pointed to the variation in optimum temperature among virus strains as a partial explanation of variation in virulence. It seems to me that these phenomena can also explain some of the observed effects of cold on viral infection and, indeed, may even account for the variability of observations. I suggest that one can expect to see an effect of cold on viral infection under certain circumstances. The appropriate circumstances would be when an animal (or man) is infected with a virus, or a particular strain of a virus, that has an optimum multiplication temperature a degree or two lower than the body temperature of the host. Under these circumstances the body temperature of the host could be an important controlling factor in limiting viral multiplication and in keeping the infection a mild, or inapparent, or latent one. And lowering the temperature in the right tissues (surface or internal, depending on the virus) only a degree or two could result in a markedly increased pace of viral multiplication, and thus lead to obvious aggravation or activation of the infection. Under these circumstances we would not expect to see an effect of cold unless the exposure were sufficient to bring about an appropriate drop in temperature in the right tissues. Nor need we expect to see an enhancing effect on a highly virulent virus with a temperature optimum near that of the host tissue. With certain host-virus combinations even an ameliorating effect of cold might be expected if the tissue temperature could be dropped below and maintained below the optimum temperature range of the virus. This concept of an effect of cold due to direct influence on viral multiplication processes within the host cell does not, of course, in itself exclude the possibility that stress, or other physiologic changes due to cold, may contribute to the reaction. But it seems to me that many of the demonstrations of an effect of cold on viral infection can be accounted for on the basis of a direct effect of tissue temperature on intracellular reactions.

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## DISCUSSION

CAMPBELL: It seems to me that the enzyme system shown in Figure 1 is an unusually sensitive system as far as kinetics go. If you were to plot the effect of temperature on your enzyme reaction, what would you find?

METCALF: We have done this repeatedly; I would say possibly 30 or 40 times.

CAMPBELL: I am not questioning the results. It's just so unusual. Earlier this afternoon somebody talked about the sensitivity of viruses to temperature as far as susceptibility goes, so this is a rather unusual reaction. Didn't you notice that the effect of temperature between 37° C and 20° C is really a tremendous difference in the activity of your enzyme?

METCALF: In other words, Dr. Campbell, you think that this is more than would seem to be indicated by experience with other systems?

CAMPBELL: Well, it's a little different, I would think. It would be really interesting to do a kinetic study on it.

METCALF: All I can say is, while we haven't performed precise kinetic studies, we have examined the reaction over a period extending from zero hours through 24 hours. We find that there is a linear release of neuraminic acid for twenty to thirty minutes, after which the rate of release slows down greatly, but does continue for eighteen to twenty-four hours. The initial effect of virus dilution upon the release of neuraminic acid is overcome at the end of twenty-four hours. The effect of temperature seems to consist of a retardation of the reaction with less neuraminic acid split off.

CAMPBELL: That will be an interesting system to study thermodynamically.

WALKER: I have been trying to relate this to some work that Dr. Billie Padgett and I have done. Dr. Padgett approached the question of the role of the enzyme of influenza a little differently, and actually aimed at selecting a strain of influenza B virus that was different in its enzymatic characteristics. She was able to get a line of virus that is quite different in its enzymatic characteristics from the parent strain in that its peak zone of activity is at about $35^{\circ}$ C, whereas the parent strain has its peak activity at about $37^{\circ}$ C. The enzymatic activity of this virus is markedly inhibited at temperatures above $35^{\circ}$ C, particularly if calcium ion is removed from the medium. She has made an effort to use temperature as a means of cutting the multiplication cycle and seeing where the effect of this enzyme may be. The parent and variant viruses appear to be very similar in all other respects, antigenic make-up, general characteristics, and so on, except for their temperature optimum for enzymatic activity. She finds that the time at which elevation of temperature will affect the multiplication cycle of the variant virus is only toward the end of the cycle, suggesting again that the enzyme is not particularly important in penetration. Elevation of temperature with this variant virus early in the cycle doesn't have much effect, but late in the cycle it does.

We also have followed the appearance of enzyme and virus in the cell, but we have interpreted the results a little differently. I am a little reluctant to think that the enzyme necessarily has an

effect upon the cell. Our interpretation has been that as multiplication goes on and virus is produced possessing enzymatic activity, you see then the appearance of enzymatic activity in the cell. This is associated with cell damage, but it would be difficult to decide which is cause and which is effect.

METCALF: Well, this is perhaps true, but in our case, every time that we domonstrate enzymic competence and show this by enzyme fabrication, we experience a corresponding reduction in the exhibitor content and there is demonstrable damage to the cells. I agree that it is difficult to separate cause and effect, but enzyme fabrication and cell damage are intimately related. Perhaps this would be a compromise between the two viewpoints.

WALKER: Schlesinger, too, has shown cyclic waves of virus level and substrate levels in the cells.

ANDREWES: Dr. Sulkin, you were quoted by Dr. Metcalf. Have you got any comments to make?

SULKIN: No, not particularly, because when those experiments were done back in 1941 and 1942, we knew nothing about neuraminidase, inhibitors, and so forth. It was sort of a naive experiment.

ANDREWES: We were all naive in those days.

SULKIN: The results have been subsequently duplicated by other workers.

CAMPBELL: Frank Lanni has done quite a bit on this inhibitor. Didn't he come up with a chemical nature of it and do some kinetic studies?

METCALF: Lanni[1] studied the interaction between enzymatically active swine influenza and egg white inhibitory mucoprotein. He described the kinetics of inhibitor inactivation, basing his

1 Lanni, F., and Y. T. Lanni. 1955. Virology 1: 40-57.

interpretation of the data upon Gottschalk's model of the structure of urinary mucoprotein.[2,3] The inhibitor reduction method of assay was used in these studies. There was no attempt to define the chemical structure of inhibitor.

2 Gottschalk, A. 1952. Nature 170: 662-663.

3 Gottschalk, A. 1954. The Blakiston Co., New York.

# THE INFLUENCE OF COLD ON VIRUS INFECTIVITY

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ABSTRACT

This study was on the characteristics of influenza $A_2$ strains which might play a role in facilitating virus invasion of host cells, and the effect of cold upon the invasive process. Strains of influenza $A_2$ selected for study were isolated from fatal cases of influenza in humans. The strains were examined for mouse toxicity following intravenous injection, cytotoxicity in HeLa and L-cells, mouse and chick embryo $ID_{50}$ values, and neuraminidase activity. Virus enzyme action was singled out for special attention on the basis of its constant association with $A_2$ strains. The enzyme activity was determined (1) by means of thiobarbituric acid analysis for free neuraminic acid, and (2) by reduction of the hemagglutination titer of mucoid inhibitor. Substrates used included neuraminmucoid from edible birds nest; neuraminlactose from bovine colostrum, and ovomucin from hens eggs. The rate and extent of enzyme action exhibited by virus showed a progressive decline as the temperature was lowered from 37° C to 4° C. A comparison of enzyme activity, virus titer, and inhibitor concentration in chick embryos at 37° C and 20° C showed corresponding decreases in enzyme activity and virus titer while inhibitor remained virtually unchanged at lower temperatures. Infection of mice was followed by extensive lung damage and death at 4° C. Considerably less damage and fewer fatalities within the test period were found at 20° C. Contrary to the chick embryo experience, the enzyme activity and virus titer showed increases at the lower temperature. Cell monolayers of monkey or hamster kidney were used in conjunction with fluorochrome analysis to follow the course of cell invasion by virus at 37° C and 20° C. Virus was first demonstrated in the cytoplasm around the nuclear membrane at 6 hours following incubation at 37° C. Virus presence in the cytoplasm was shown for at least 72 hours. The course of invasion at 20° C was different. Virus was first detected after 10 hours. It appeared in the cytoplasm, but failed to show a significant increase in numbers. It was possible to show virus accumulating at the periphery of allantoic membranes within 12 hours when embryos were incubated at 37° C. No virus accumulation was observed in membranes from embryos incubated at 20° C. Single-caged mice pre-exposed and maintained at 4° C showed massive virus invasion of lung and bronchi, while group-caged mice showed only minimal virus invasion.

The effect of cold upon the course of influenza virus infections represents a special problem in host-parasite relationships. Is the primary influence of temperature a factor altering the biology of a host, or is it directed against the virus? Which is of greater impor-

tance, physiological patterns of the host, or virus infectivity?

This study was concerned with the initial stage of virus-cell interaction using influenza virus with selected hosts as the experimental model. The influence of low temperature upon the interaction has been examined from the standpoint of its effect upon virus infectivity.

The proposal that neuraminidase facilitates the penetration of a host cell by influenza virus presumes that infectious virus should possess enzyme activity. This viewpoint has been presented by Gottschalk (1957) who believes enzyme action renders cell wall mucoproteins more permeable to virus and thereby facilitates virus penetration of a host cell.

This study began with the knowledge that experimental influenza infections have been represented as occurring in the absence of enzyme action (Fazekas, 1948; Fazekas and Graham, 1949). Without adopting a position on the essentiality of enzyme to the infectious process, it was reasoned that clues to the influence of cold upon virus infectivity might be gained by a consideration of the effect of cold upon neuraminidase production by influenza virus.

## THE EFFECT OF COLD UPON THE NEURAMINIDASE ACTIVITY OF INFLUENZA VIRUS

Enzyme action was measured by two methods. The thiobarbituric acid (TBA) method of Warren (1959) was used to determine the free neuraminic acid resulting from combination of virus and a neuraminic acid containing substrate. A neuraminmucoid substrate was prepared from oriental "edible birds nest" by a modification of the method of Lawton et al. (1956). Edible birds nest is a salivary mucoid produced by the Collocalia species of swift. The second method included the use of ovomucin obtained from hens eggs (Gottschalk and Lind, 1949) in an inhibitor reduction (IR) titration following combination of virus and ovomucin (Isaacs and Edney, 1950).

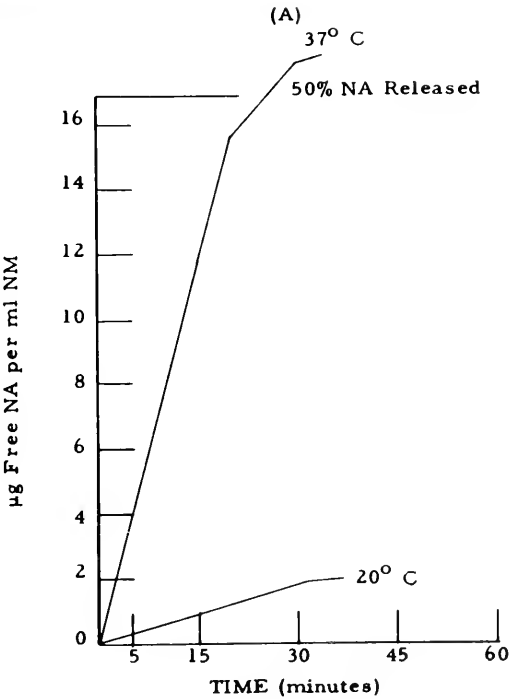


Figure 1a. The release of neuraminic acid from mixtures of influenza virus (A2/Jap 305/57) and neuramin mucoid at 37° C, 20° C, and 4° C.

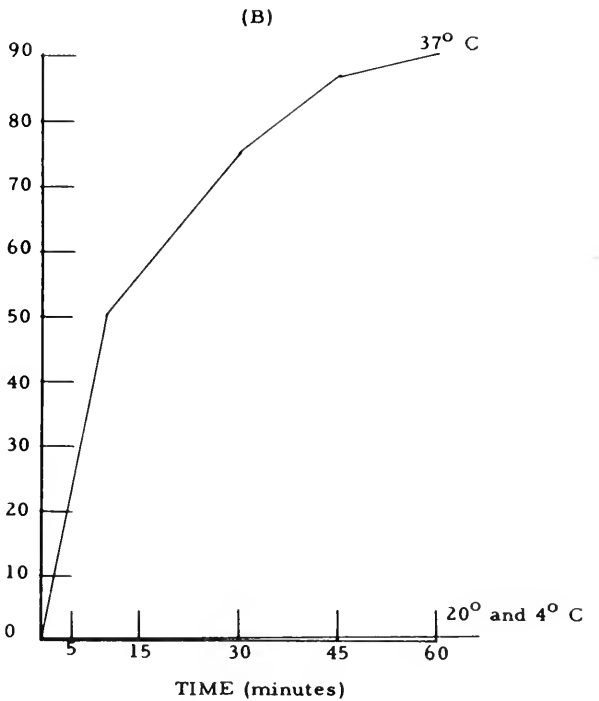


Figure 1b. The per cent split product from enzymic action of influenza virus (A2/Jap 305/57) on neuramin mucoid at 37° C, 20° C, and 4° C.

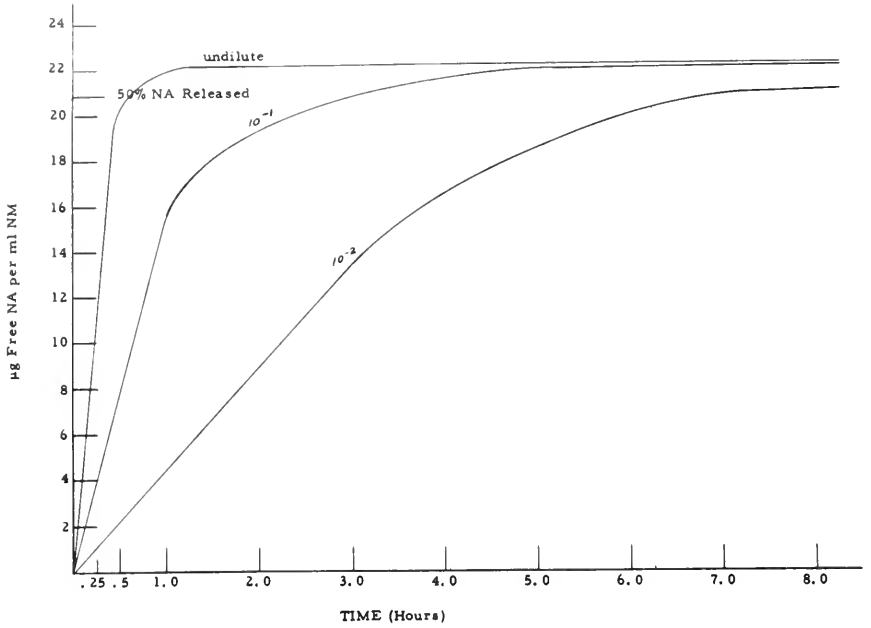


Figure 2. The effect of neuraminidase concentration upon the release of neuraminic acid from mixtures of influenza virus (A2/Jap 305/57) and neuramin mucoid at 37° C.

Using neuraminmucoid and enzyme mixtures incubated at 37° C, free neuraminic acid was linearly split off during the first 20 minutes. When the same mixtures were combined and incubated at 20° C a slow linear increase in free neuraminic acid was observed during the 30 minute test interval. No reaction took place within 30 minutes at 4° C. The use of the IR method for measurement of enzyme action confirmed the findings of the TBA analyses. Differences observed in the results obtained by the two methods were indicative either of a different order of sensitivity, or a difference in the substance being measured. For example the IR titration gave a yield of approximately 90 per cent split product compared to about 53 per cent for the TBA method. Enzymic action at 20° C demonstrated by TBA analysis was not shown by the IR method.

The concentration of enzyme affected the release of neuraminic acid as shown in Figure 2. Not only was the rate of release proportional to enzymic concentration, but also to the total amount released. The same result using other substrate materials at 37° C

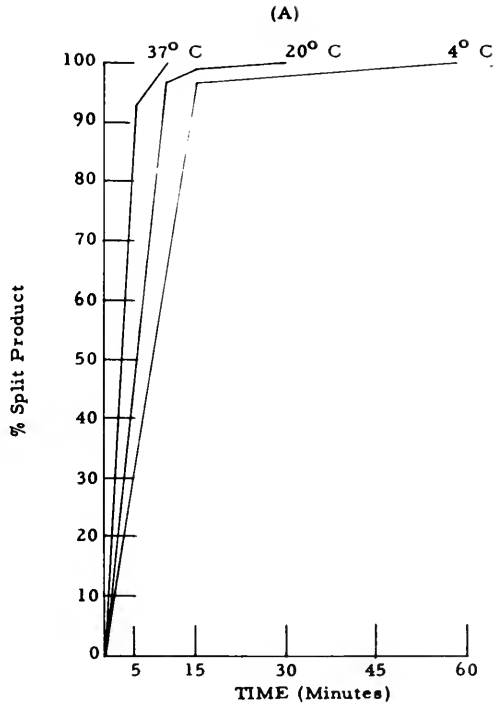


Figure 3a. The effect of temperature upon the enzymic action of influenza virus (A2/ Jap 305/57) on ovomucin measured by inhibitor reduction.

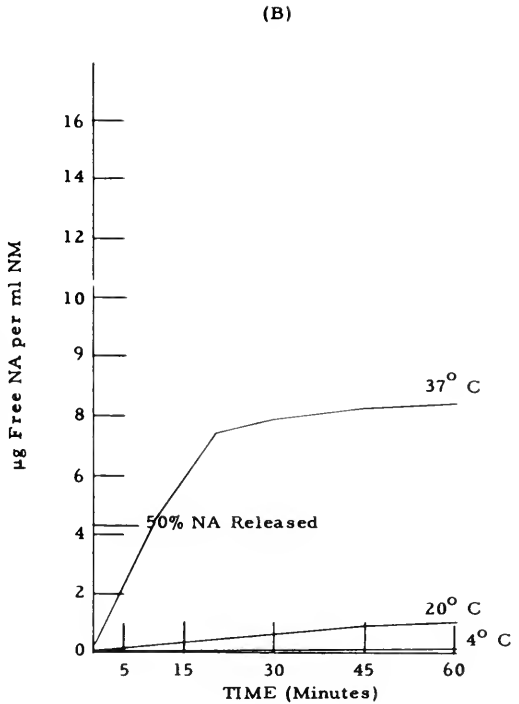


Figure 3b. The effect of temperature upon the enzymic action of influenza virus (B2/Jap 305/57) on ovomucin measured by thiobarbituric acid method.

was reported recently by Noll, Aeyagi and Orlando (1961) and Mayren et al. (1961).

When ovomucin was used in place of neuraminmucoid, different results were obtained. Enzymic destruction of substrate occurred rapidly at all temperatures when measured by the IR method. Only slight differences in the rate of of substrate hydrolysis and the time required to reach the same endpoint could be shown. The results of the TBA analyses showed the rate of enzymic attack on ovomucin to be similar to the values found with neuraminmucoid. The efficiency of the hydrolysis was considerably improved, however, and indicated a more labile, more available enzyme-vulnerable structure (Fig. 3).

The inhibitory titer of the two inhibitor preparations for indicator virus varied markedly. Based on the bound neuraminic acid content, ovomucin containing $3 \times 10^{-4}$ micrograms was inhibitory. Using the same basis neuraminmucoid containing $7 \times 10^{-8}$ micrograms was inhibitory. The ovomucin inhibitory value of $3 \times 10^{-4}$ micrograms agreed well with the value $4\text{-}8 \times 10^{-4}$ micrograms reported by Gottschalk and Lind (1949) for their preparation of ovomucin. Tamm and Horsfall (1952) reported an inhibitor titer of $3 \times 10^{-4}$ for urinary mucoprotein at equilibrium with influenza virus. There are no values published for neuraminmucoid.

Ovomucin contained 0.18 per cent total protein, determined by the Biuret test; 0.05 per cent hexose, determined by the modified Orcinol method of Rosevear and Smith (1961); and 1.5 micrograms per ml of total bound neuraminic acid. The neuraminmucoid preparation contained 4.86 per cent total solids, 1.68 per cent total protein, 0.97 per cent hexose, and 11 micrograms per ml of bound neuraminic acid.

For the purposes of the study, the results showed that neuraminidase continued to attack substrate at 20° C, but with decreased efficiency. Both the rate and extent of enzyme action were affected. The marked difference in the values obtained with ovomucin and neuraminmucoid substrates was interpreted as the result of chemically different compounds possessing enzyme-labile structures of varying accessibility and possibly different chemical composition.

# SIMULTANEOUS MEASUREMENTS OF VIRUS, INHIBITOR, AND ENZYME LEVELS DURING INFLUENZA INFECTIONS

In order to examine the effect of low temperature upon virus infectivity, studies were directed toward the relationships existing between virus multiplication, enzyme production, and inhibitor levels in the infected host. The relationships were determined first at the normal environmental temperature of the host and secondly at subnormal temperatures. Both the chick embryo and white mouse were used as experimental hosts. Special attention was given to A2 strains, several of which had been isolated from fatal human cases of influenza.[1] In addition, A and A1 strains were used. The enzyme activity of the different strains varied from the A1 which possessed low reactivity, to the A strains which varied from low to intermediate reactivity, to the A2 strains which had the greatest activity.

A typical experiment using the chick embryo was conducted as follows. Ten-twelve day embryos were injected via the allantoic fluid with 0.1 ml volumes of virus of known concentration. A similar number of control embryos were injected in the same way with the same volume of diluent. Control and test embryos were incubated at 36° C to 37° C and 20° C. Six to ten embryos from each group were removed at regular intervals following injection, the allantoic membranes were harvested, washed, and weighed, and 50 per cent suspensions were then prepared. Aliquots of the suspensions were rapidly frozen and stored at -70° C. The membrane suspensions were examined for their virus content by means of chick embryo $LD_{50}$ titrations. The enzyme content was determined by TBA or IR assay, and the inhibitor level was measured using indicator virus.

The result of a typical experiment conducted at 37° C using A2 strains was as follows. Virus multiplication was indicated by a steady increase in detectable virus after 1 hour. Beginning at 4 hours the inhibitor content of the membranes began to decline and con-

1 Obtained through the courtesy of Dr. R. Q. Robinson, International Influenza Center for the Americas, Communicable Disease Center, US Public Health Service, Atlanta, Georgia, and Dr. A. F. Rasmussen, Jr., University of California Medical Center, Los Angeles, California.

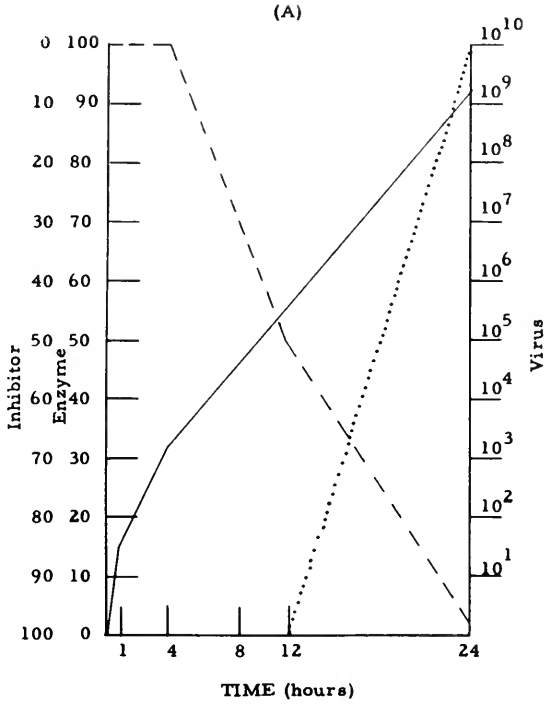


Figure 4a. Virus multiplication, enzyme production and inhibitor content in influenza (A2/Jap 305/57) infected membranes at 37° C. ---- Virus concentration (log $CEID_{50}$); ---- Inhibitor content (% reduction inhibitor); . . . . Enzyme production (% split product).

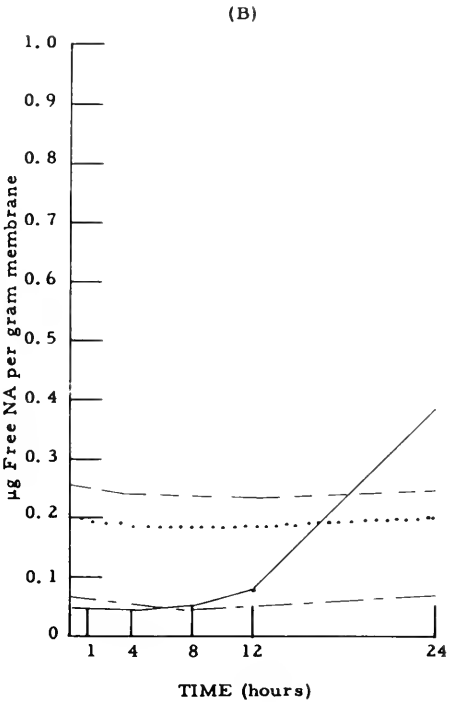


Figure 4b. Enzyme synthesis in influenza (A2/Jap 305/57) infected membranes at 37° C. ——— Allantoic membrane (AM) - test; – – – – – AM - Control. – – – – Allantoic fluid (AF) - test; . . . . AF - Control.

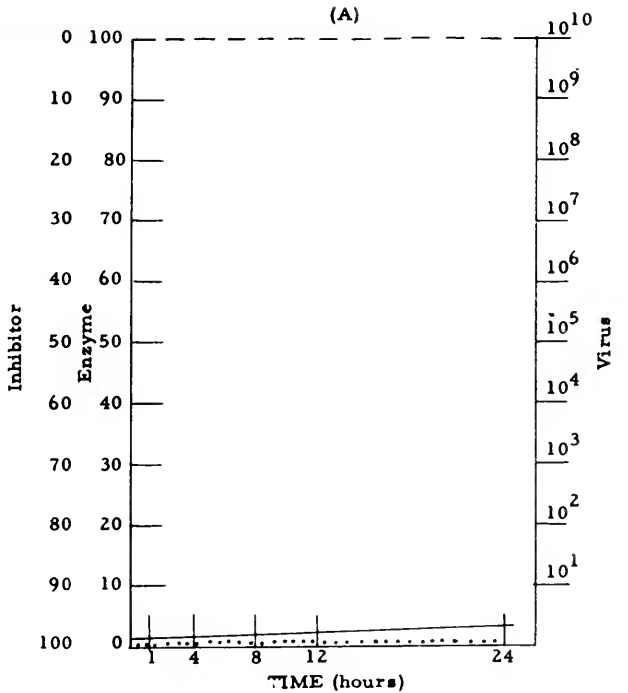


Figure 5a. Virus multiplication, enzyme production and inhibitor content in influenza (A2/Jap 305/57) membranes at 20° C. ——— Virus concentration (log $CEID_{50}$); ----- Inhibitor content (% reduction inhibitor); . . . . Enzyme production (% split product).

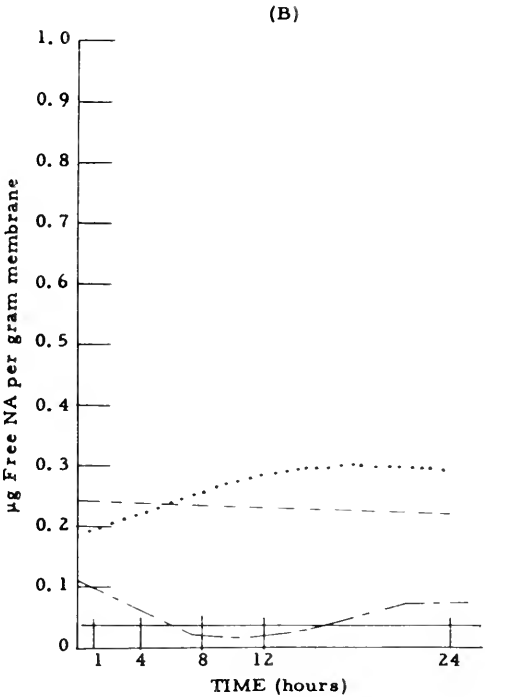


Figure 5b. Enzyme synthesis in influenza (A2/Jap/57) infected membranes at 20° C. ——— Allantoic membrane (AM) - test; — — — AM - Control; ----- Allantoic fluid (AF) - test; . . . . . AF - Control.

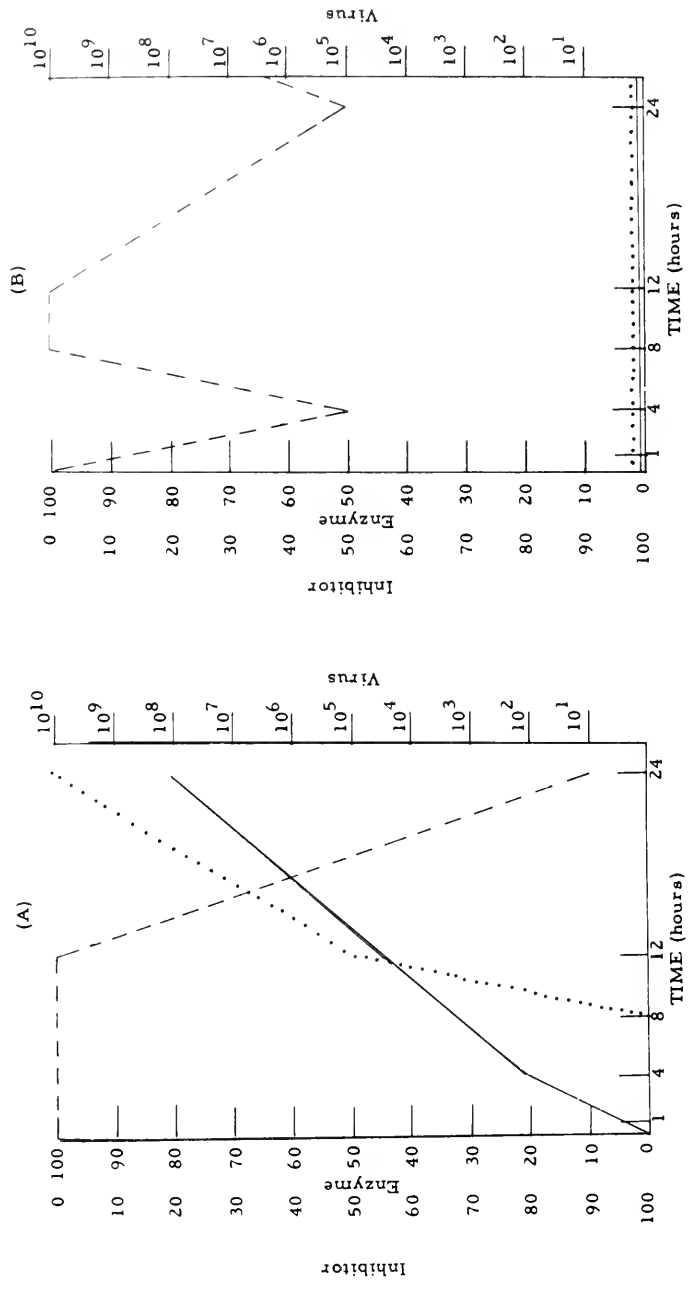


Figure 6a. Virus multiplication, enzyme production and inhibitor content in influenza (A/PR-8/34) infected membranes at 37° C. —— Virus concentration (log $CEID_{50}$); ---- Inhibitor content (% reduction inhibitor); ..... Enzyme production (% split product).

Figure 6b. Virus multiplication, enzyme production and inhibitor content in influenza (A/PR-8/34) infected membranes at 20° C.

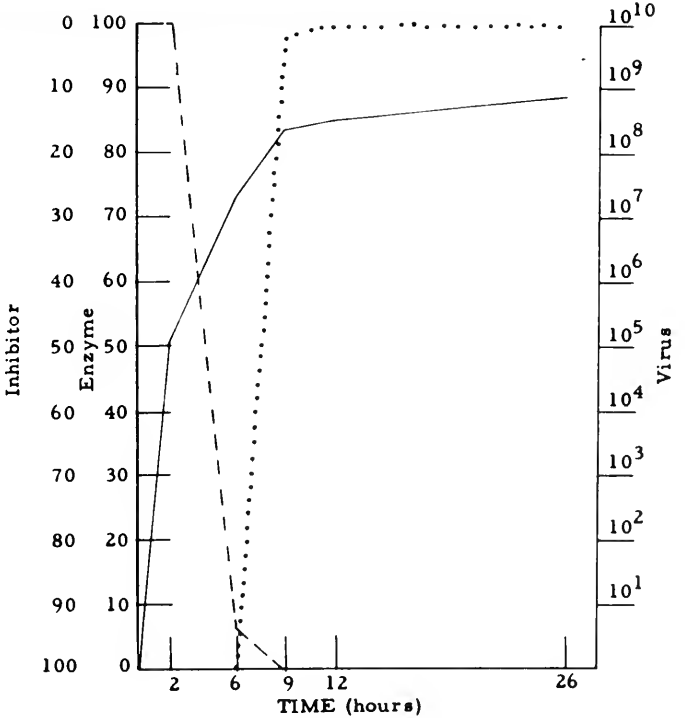


Figure 7a. Virus multiplication, enzyme production and inhibitor content in influenza (A/PR-8/34) infected membranes at 37° C.

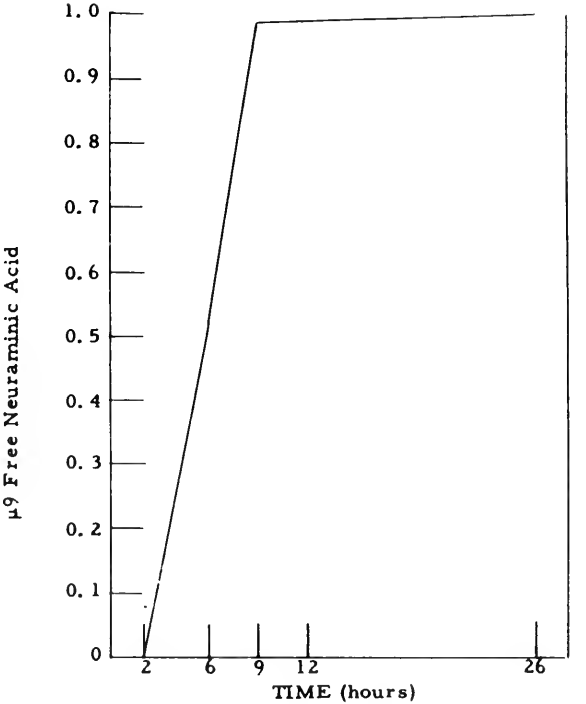


Figure 7b. Neuraminidase synthesis in influenza (A2/Jap 305/57) infected membranes at 37° C. ——— Virus concentration (log $CEID_{50}$); ----- Inhibitor content (% reduction inhibitor); . . . . . Enzyme production (% split product).

tinued to fall throughout the 24 hour test period. Sometime after 12 hours the production of enzyme within the membranes was detected, with a steady increase shown during the remainder of the test period. Confirmation of the fabrication of enzyme within the infected allantoic membrane was obtained in separate experiments by detection of a rise of free neuraminic acid as measured by the TBA assay. The level of free neuraminic acid in infected membranes began to increase at 12 hours after infection and continued to increase through 24 hours. No increase could be shown for the non-infected membranes (Fig. 4).

When experiments of this kind were conducted with A2 strains in chick embryos incubated at 20° C, the following results were found. There was no significant increase in the virus concentration of membranes, no decrease in inhibitor level, and no enzyme formation was detected. Again there was agreement between inhibitor reduction titrations and TBA assays (Fig. 5).

The findings obtained in chick embryos with A2 strains possessing a high enzyme activity were next compared to the results found with a PR-8 strain of low enzyme activity. Virus multiplication and enzyme production occurred, and the inhibitor level decreased accordingly when the experiment was conducted at 37° C. At 20° C no virus growth or enzyme production was detected. The inhibitor level fluctuated but did not show a steady decline (Fig. 6).

Throughout the study a virus inoculum of 1000 $LD_{50}$ doses was used. It was decided to determine the effect of increasing the doses given in the inoculum upon the relationships of enzyme production and inhibitor content. Accordingly, an inoculum of $10^7$ $LD_{50}$ doses per embryo was used. The net result was an accelerated appearance of enzyme and earlier decline of inhibitor. The virus content of membrane reached an early peak accompanied by a rapid rise in enzyme production between 6 and 9 hours. Membrane inhibitor fell rapidly between 2 and 6 hours (Fig. 7).

The pattern of events emerging from these experiments confirmed the existence of a direct relationship between virus multiplication and the appearance of enzyme activity in the allontoic membrane.

Nell et al., (1961) reported similar findings in an earlier publication which dealt with infected chick embryos. Accompanying the rise in virus titer and enzyme content was a corresponding drop in the concentration of membrane inhibitor. This finding tended to support the contention of Isaacs and Edney (1950) who indicated a role for intracellular receptors in influenza infections on the basis of a correlation between the effectiveness of indicator viruses as interfering agents and their position in the "inhibitor gradient" of allantoic membrane extracts. The time of appearance of enzyme depended on the speed of virus growth. When $10^3$ $LD_{50}$ doses were given, enzyme formation was usually detected after 12 hours. If the number of $LD_{50}$ doses administered was increased to $10^7$, enzyme formation was observed between 6 and 9 hours. These results were in agreement with previously reported findings on virus multiplication by Henle (1953) and Ackermann and Francis (1954). When the environmental temperature was decreased to 20° C, virus growth failed to materialize, no enzyme was fabricated and the inhibitor level remained unchanged.

Once the relationships of enzyme fabrication, virus growth, and inhibitor decline had been shown in embryos, attention was directed to these same considerations in influenza infected mice. Albino mice were infected intranasally with mouse adapted A2 and A1 strains.[2] Two groups of mice were established, one group-caged at the normal animal room temperature of 20° C, and the other at 4° C. The mice placed at 4° C were individually caged in plastic containers without bedding material. Water was added to the containers in amounts adequate to give a thin discontinuous film of moisture covering the floor. The mice were maintained at 4° C until signs of distress were noted. These included shivering, ruffled fur, apathy to stimuli, and blanched ears. Mice from the two groups received an inoculum previously determined to give 50 per cent lung consolidation at 48 hours in mice maintained at 20° C. An equal number of control mice from the two groups received an inoculum of sterile saline. Further control mice were inoculated to determine the 50 per cent lung consolidation endpoint. Mice from test and control groups at 20° C and 4° C were sacrificed at 0, 8, 24 and 48 hours. Lungs and trachea were examined for their virus titer, enzyme, and inhibitor content.

2 Obtained through the courtesy of Dr. T. Francis, Jr., School of Public Health, University of Michigan, Ann Arbor, Michigan.

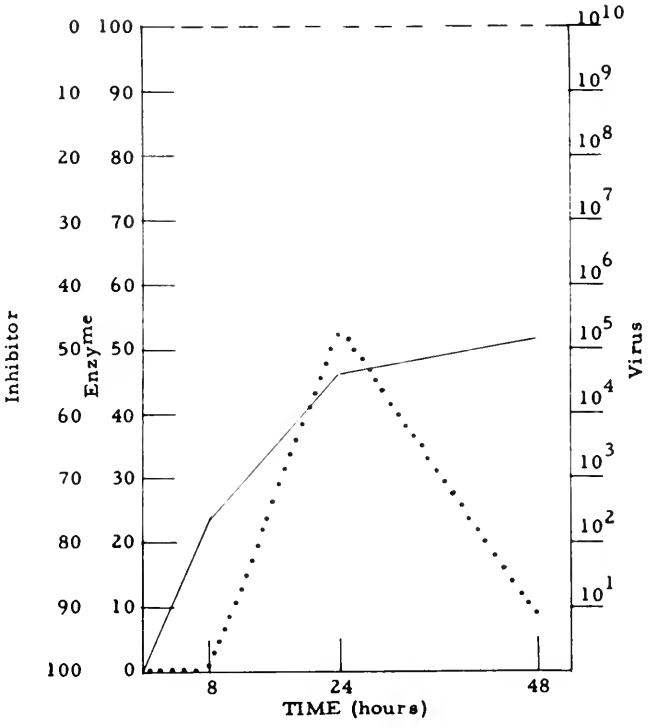


Figure 8a. Virus multiplication, enzyme production and inhibitor content in influenza (A2/Ann Arbor/58) infected mouse lungs at 20° C.

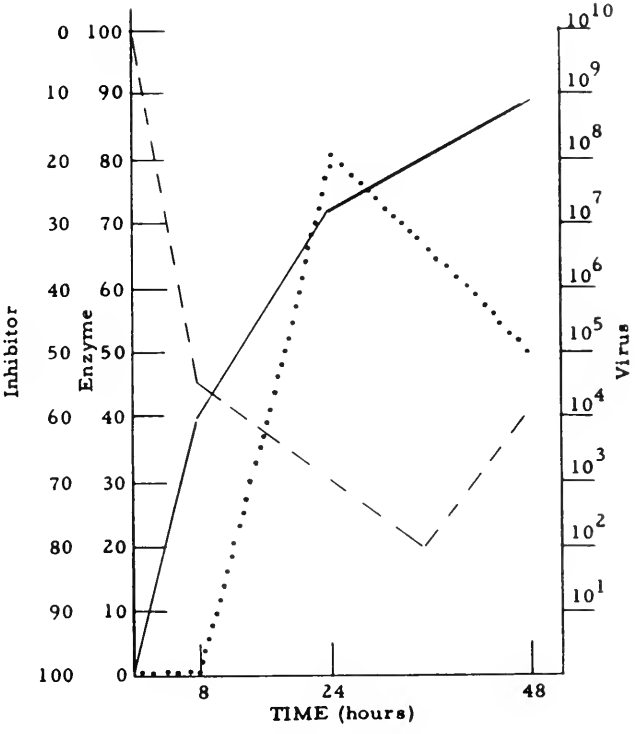


Figure 8b. Virus multiplication, enzyme production and inhibitor content in influenza (A2/Ann Arbor/58) infected mouse lungs at 4° C. ——— Virus concentration (log $CEID_{50}$); ----- Inhibitor content (% reduction inhibitor); . . . . Enzyme production (% split product).

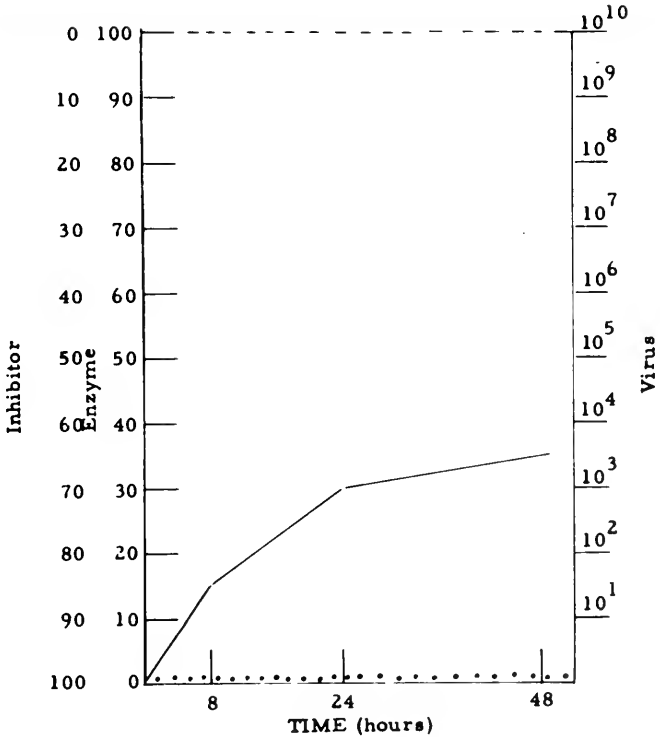


Figure 9a. Virus multiplication, enzyme production and inhibitor content in influenza (A1/FM-1/47) infected mouse lungs at 20° C.

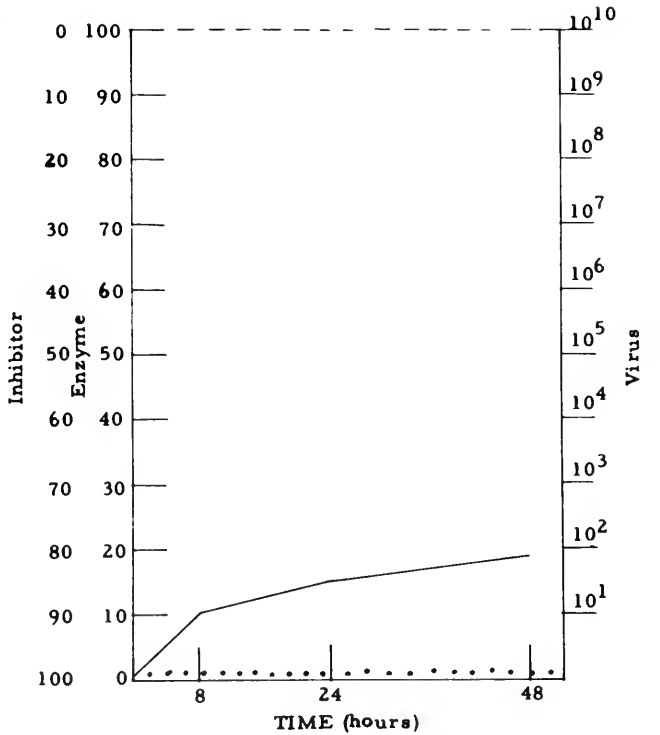


Figure 9b. Virus multiplication, enzyme production and inhibitor content in influenza (A/PR-8/34) infected mouse lungs at 20° C.— Virus concentration;---Inhibitor content (% reduction inhibitor); . . .Enzyme production (% split product).

Gross macroscopic examination of the lungs showed complete consolidation to have occurred in the mice kept at 4° C while 50 per cent consolidation was found in lungs removed from mice kept at 20° C. Enzyme production occurred in both groups, but inhibitor levels declined only in the cold exposed mice. A cold environment, therefore, led to a more rapid growth of virus which in turn led to a greater yield of enzyme and a decline in inhibitor content (Fig. 8).

The importance of neuraminidase activity to cell damage was shown by the results obtained following inoculation of mice with A1 and A strains having no detectable enzyme action. Virus multiplication was limited, no enzyme was produced and there was no loss of inhibitor. The lungs removed at sacrifice were normal in appearance and no cell damage could be demonstrated (Fig. 9).

## FLUOROCHROME STUDIES OF EXPERIMENTAL INFLUENZA INFECTIONS

Host response to virus infection at the cellular level was studied with the help of the fluorochrome acridine orange. Monkey kidney monolayers were infected with A2 strains of high and low enzyme activity at environmental temperatures of 20° C and 37° C, and examined at intervals up to 5 days. "Flying coverslips" were fixed in chilled absolute methyl alcohol, hydrated, and stained 5 to 9 minutes with a 1 to 10,000 dilution of acridine orange at a pH of 3.8. The coverslips were examined with a Fluorestar microscope used in conjunction with an Osram HBO-200 illuminator. The exciter filter used was a Corning No. 5860, half-thickness filter. Photomicrographs were made using Ektachrome, type B film.

Non-infected monolayers showed the green fluorescing nuclei characteristic of DNA material, with yellowish-white nucleoli. The cytoplasm exhibited a rust color symptomatic of RNA. Six hours after infection the cytoplasm around the nucleus began to show a brighter rust fluorescence. By 18 hours the fluorescence became a bright orange-red color and could easily be distinguished. At 30

hours cytoplasmic inclusions were observed. From 72 to 96 hours the infected cell showed extensive cytopathogenic change. The specificity of the stain was controlled by the use of RN'ase which made it possible to distinguish the presence of nonspecific staining.

The sequence of events in the invasion of cell monolayers did not differ noticeably at 37° C or 20° C. The presence of virus, its intracytoplasmic growth, cytopathology, and cell destruction, could not be differentiated with certainty at the two temperatures.

Examination of allantoic membranes was accomplished by means of paraffin sections prepared after the method of Coons and Sainte-Marie (1960). Three to five micron sections were cut on a rotary microtome and stained with acridine orange. Virus growth at 37° C was shown to occur predominantly in the entodermal cells lining the allantoic cavity, although some virus staining material was found in the ectoderm. Infected membranes kept at 20° C showed practically no virus staining material in the entodermal cells.

When sections of mouse lung were examined, the extent of virus invasion was shown. A section of non-infected mouse lung failed to show virus-staining material. Sections of lungs from mice infected at 20° C showed virus-staining material gathered around the bronchi as well as scattered throughout the lung. Sections from infected mice of the 4° C group showed a more massive accumulation of virus-staining material. Greater concentrations of virus appeared immediately adjacent to the bronchi.

The results of the fluorochrome examinations furnished visual evidence that the neuraminidase-active strains of influenza used in the study found their way into host cells of chick embryos, monkey kidney monolayers and mice. The initial stage of cell invasion was not affected by the degree of enzyme activity associated with the A2 strains. Thus, strains of high enzyme activity could not be shown to possess a penetration advantage over strains of low enzyme activity.

## DISCUSSION

The measure of infectious virus used in the study was the ability to multiply sufficiently within a host cell to damage it by means of enzymic attack. The direct relationship shown between cell damage and neuraminidase production led to this conclusion. Proceeding on this basis, the results indicated that cold had no effect per se upon the infectious quality of virus. For example, the penetration of a host cell was not affected, and the in vitro effect upon neuraminidase activity was minimal. Instead, cold was shown to exert an influence on the host-parasite relationship subsequent to virus penetration. The findings suggested that cold acted to alter some host structure important for defense and accordingly render the cell more vulnerable to enzyme action. It was postulated that the structure affected by cold was mucoprotein in nature. This viewpoint would represent a modification of Gottschalk's enzyme penetration theory (1957). The important difference lies in the relationship of enzyme activity to cell damage in the present study, rather than cell penetration.

The results were an extension of the findings of Sulkin (1945) who reported a more serious infection in cold exposed mice with little change in mortalities. The fatalities occurring in the present study were attributed to a combination of enzymic competence of the virus strains used and physiologic insult sustained by the mice.

The findings of the present study constituted an interesting contrast to the results reported by Sarracino and Soule (1941). These authors suggested that infection depends on the amount and virulence of virus rather than the general resistance of the host. My study indicated that virus possessing enzyme activity was infectious and thus virulent. It also showed that the severity of influenza infections was not the exclusive result of virus virulence, but could be conditioned by cold-induced physiologic alterations within a host.

An attempt at translation of the results of the present study in terms of the "winter factor" of Andrewes (1958) would be presumptuous. The results did suggest, however, that a study of physiologic alterations of mucoprotein structures of a cell exposed to virus

possessing enzymic competence might provide clues to the nature of the winter factor.

## SUMMARY

Infectious influenza virus was considered to be characterized by an enzymic potential capable of causing host cell damage. No effect of cold upon infectious virus per se could be shown. Any influence exerted by cold upon experimental influenza infections was shown to be closely related to a host-induced effect. Host cell damage was believed to follow enzymic action upon muco-protein structures of the cell.

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## DISCUSSION

REINHARD: Dr. Walker, what is the temperature of a baby mouse?

WALKER: I don't know, but I would like to. This brings up a very interesting point. I indicated that we have begun work involving using both tissues of baby mice and adult mice. It is quite evident that raising the body temperature of a baby mouse does not produce the same effects that I showed here for the adult mouse. We have exposed infant mice to incubator temperatures up to about 35° C. Above this temperature, the infant mice do not survive. I think perhaps the baby mice could tolerate higher temperatures, but the mother refuses to feed them or her lactation stops at higher temperatures. She apparently has enough troubles at that temperature without having these little furnaces hanging on her, so she retreats to the opposite side of the cage and the mice are not fed. I don't know what the temperature of baby mice is at ambient temperatures of 35° C, but I think it is probably higher than normal. I haven't succeeded, so far, in finding a probe that will allow me to measure the body temperature of a forty-eight-hour-old infant mouse.

One other thing should be pointed out, too, and that is that the pancreas appears to be different from other tissues. Coxsackie virus will multiply in the pancreas of the adult mouse even at

ordinary temperatures, but one can raise the body temperature sufficiently so that even this multiplication is choked off.

NUNGESTER: This pancreas thing intrigues me very much.

WALKER: It intrigues me, too, because there are other viruses that seem to multiply well in the pancreas.

NUNGESTER: But by and large, the pancreas is not susceptible to bacterial infection, at least as far as I know.

WALKER: But I think it is susceptible to a number of viruses.

REINHARD: I might offer the information of some recent gastroenterological research which indicates that the pancreas has different proclivity for amino acid uptake. Certain kinds of amino acids are readily absorbed by the pancreas, and this might be one of the differences causing increased tissue susceptibility.

WALKER: So far, we have had great difficulty in trying to get it to persist or multiply in culture.

METCALF: Dr. Walker, I think you have a very interesting theory. In view of your results as well as the work of Dubos and Wenner,[1] I wonder if you are forced to describe a polydisperse virus population with respect to genetic variance. Are you selecting one member of a polydispersed population at a given temperature?

WALKER: I am not any more worried with that than with any other explanation of multiplication at any temperature. I think temperature is going to be a selective force on virus populations, and I think that, as Lwoff has shown, it is possible to isolate strains that are capable of multiplying at higher temperatures. This is part of the basis for his contention that temperature is related to virulence. Is this pertinent to your question?

1 Dubes, G. R., and H. A. Wenner. Virology 4: 275-296.

METCALF: I wondered if you felt this to be a condition which might prove deleterious to your theory.

WALKER: No, I think that this is reasonable. This is just another selective pressure, and we will have the same problems with selection that will apply to any other explanation of the effect of temperature on infection.

CAMPBELL: I might point out that it is pretty well known that in neonatal animals there is a question whether they produce any antibodies at all so that when you expose them to antigen, they may actually exhibit a so-called tolerance or immune paralysis. Their immune mechanism is not working at this point. You had an exposure of six days at 25° C.

WALKER: And then a shift to cold.

CAMPBELL: Yes, and then in this case, you didn't have any effect.

WALKER: Right.

CAMPBELL: I was wondering whether you thought that this exposure, some way or another, actually causes an adaptation.

WALKER: The point was, if they were held at ordinary room temperature for as long as six days after receiving virus, then the shift to 4° C had no effect, but after only four days, the cold still caused high mortality. My explanation or speculation is this; by six days, the virus has been reduced to very low levels in practically all of the tissues except the pancreas. By that time, there is still a lot of antibody in the blood, and although virus activity might be increased in the pancreas, there would be no opportunity for it to be disseminated widely to other tissues that would be made more susceptible by the lowered temperature. The virus must be widely spread to cells before antibody appears in the blood, and the exposure to cold must occur while there is still a sufficient quantity of virus in the tissues. I would expect in most of these tissues that the virus is pretty well gone by six days.

PREVITE: Your findings are parallel in some ways to those that I have reported on endotoxin. If cold exposure was delayed too long, the animal had manipulated this poison somehow so that he was no longer sensitized to it by cold stress.

WALKER: Then your test material is gone.

PREVITE: Correct.

SULKIN: Dr. Campbell called attention to the fact that the infant mouse does not produce antibody. Coxsackie viruses are unique among viruses in that they elicit an antibody response very, very, rapidly. In man, for example, antibodies are demonstrative very early after first signs of infection. Yet a week-old mouse will produce antibody, whereas the one-day-old mouse will not. Overland showed that the maturation of the antibody forming mechanism in the animal accounts for this rapid acquisition of resistance to infection with Coxsackie viruses.

MITCHELL: Will someone please define for me this infant mouse? Don't say baby; I know that.

WALKER: In Coxsackie B-1 virus infection, for instance, the mouse is most sensitive to the virus up to about forty-eight hours in age. Large inocula of this virus will cause lethal infections in mice up to about eight days of age, but then there is a very abrupt cut-off with no deaths occurring in mice inoculated at an older age. But the mice are most susceptible to small inocula in their first forty-eight hours after birth.

MITCHELL: I was interested in those extra days of exposure; after this delay, the animal would be ten, eleven, or twelve days old.

WALKER: No, you misunderstood, because this exposure to cold is in adult mice. The infant mouse enters in here only because the adult mouse in the cold behaves rather like the infant mouse at normal room temperature.

PREVITE: Dr. Walker, in some of your data you mentioned

that you used ACTH, and this apparently didn't enhance viral multiplication. What was the regimen that you used and how many injections did you give?

WALKER: Four mg in gel, as I recall, given every twelve hours for a period of six days. ACTH has been shown very rarely to have an effect upon viral infections in the mouse. I don't understand why it should not. You showed the effect of ACTH upon toxin, but this may be quite a different thing. ACTH does produce physiological changes in the mouse that indicate that the ACTH is active, but the failure of ACTH to aggravate viral infections seems to be a peculiarity of the mouse. Cortisone has quite an effect upon viral infection in the mouse, but ACTH has very little.

McCLAUGHRY: Certainly the thesis that Dr. Watson has presented appeals to me very much as a person interested in human physiology because of the fact that the general pattern of interactions of related functions of cells and tissues fits the category. The modifications of a particular kind of cell reaction or tissue reaction modifies the balance among all functions, and therefore, the modification of temperature at which cells are held might very well favor certain enzymic processes which would be involved, perhaps, in replication of viral protein as compared with certain other cellular processes.

MARROW: Does the level of interferon which responds to the increase in temperature account for part of this decrease in virulence with increased temperatures?

ANDREWES: I think it may work another way. Issacs gave a review on interferon at the Montreal meeting; one of the things that he mentioned was that he has been in touch with Lwoff about this, and he feels, concerning the virulence of the strains of virus which do better at high temperatures, that perhaps they are able to do so because they become less sensitive to the action of interferon.

# SUPPRESSIVE EFFECT OF LOW ENVIRONMENTAL TEMPERATURE ON VIRAL INFECTION IN BATS[1]

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ABSTRACT

Having established the susceptibility of insectivorous bats held at 24° C to 29° C to experimental infection with rabies and encephalitis viruses and determined that virus particles introduced peripherally may invade and multiply in brown fat in addition to other tissues, studies were extended to establish the influence of low temperature on the course of these infections in these animals. Viral proliferation in interscapular brown fat, an organized, bi-lobed structure believed to be present in all hibernating animals, suggested that this tissue might provide a focus of infection or target organ from which virus could disseminate to other tissues. In view of the function of brown fat in maintaining the hibernating animal, it seemed logical to assume that virus particles present in this tissue would remain viable during periods of hibernation, sustained by the same mechanism which nurtures the whole animal, and, upon arousal of the animal would multiply and disseminate to other sites. This rationale has formed the basis for our studies on the influence of low temperature on viral multiplication in bats and the data to be presented will be drawn from the following major areas under investigation: (1) The susceptibility of various species of bats maintained at 24° C to 29° C to experimental infection with rabies and encephalitis viruses. (2) The influence of low temperature on initiation of viral infection in bats and on the course of a previously established infection in these animals. (3) The influence of low temperature on antibody formation in bats in response to bacterial and viral antigens (some information relative to rates of antigen and antibody degradation in the torpid bat will also be included). (4) Reference will be made to studies on the influence of low temperature on viral multiplication in monolayer and explant cultures of tissues of the bat as compared with tissues of warm blooded animals.

1 The original work reported herein was supported by research grants from the Caruth Foundation, the National Institutes of Health, United States Public Health Service, and the Commission on Viral Infections, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army.

The isolation of rabies virus from naturally infected insectivorous bats in the United States in recent years has focused the attention of epidemiologists and virologists on the Chiroptera as still another reservoir host for this virus in nature. Since our laboratory reported the first human death believed to have been due to the bite of a naturally infected bat (Sulkin and Greve, 1954), we became interested in the association of bats with rabies and more especially in how these animals are able to survive infection with this hitherto presumed fatal viral infection. Investigators in South America and Trinidad demonstrated that the vampire bat could suffer sustained infections with rabies virus and pass the virus through infected saliva to susceptible animals without themselves showing overt symptoms or succumbing to the disease (Enright, 1956). We now have experimental evidence that insectivorous species of bats native to the United States may be infected with rabies virus and never show symptoms of the disease even though virus can be demonstrated in brain as well as other tissues (Sulkin, 1962).

## RABIES STUDIES IN INSECTIVOROUS BATS

Studies on the course of experimental rabies infection in insectivorous bats were undertaken with the view to determining the susceptibility of these animals to peripheral inoculation with rabies virus and to ascertain which tissues were involved in the infection (Sulkin et al., 1959). We have also attempted to determine the influence of certain physiological characteristics of the bat on experimental rabies infection in these animals (Sulkin et al., 1960; Sims, Allen and Sulkin, unpublished data). In this regard, the phenomenon of hibernation was the first area to receive attention.

Species of bats which inhabit temperate zones are efficient hibernators who remain quiescent for several months of the year. It has been suggested that this conservation of energy is the reason why these mammals, despite their small size, are relatively long-lived (Griffin, 1958). Also, many investigators (Rasmussen, 1923, 1924; Remillard, 1958; Johansson, 1959, 1960; Kayser, 1961) believe that

the so-called "hibernating gland", a bilobed, organ-like accumulation of brown adipose tissue found in the interscapular region of hibernating animals (and in certain non-hibernators as well), plays a role in sustaining the hibernating animal during its period of winter dormancy. Histochemical analysis of the interscapular brown fat of the little brown bat (Myotis l. lucifugus) indicates that this tissue builds up quantities of lipid during pre-hibernation months and that the various biochemical constituents of this tissue are depleted as hibernation progresses (Remillard, 1958). Studies comparing the nature of brown and white fat of several animal species indicate that brown adipose tissue is histologically distinct and physiologically more active than white fat (Fawcett, 1952). Recognition of the role of brown fat in sustaining the hibernating bat during periods of inactivity, together with reports dealing with the multiplication of Coxsackie virus (Pappenheimer et al., 1950; Grodums and Dempster, 1959) and poliovirus (Shwartzman, 1952) in the brown fat of mice and hamsters, suggested that this tissue of bats might provide a site for rabies virus sequestration in the asymptomatic host; a latent focus of infection from which virus sustained during the period of hibernation by the same mechanism which nutures the whole animal could be activated to invade and multiply in other tissues, including salivary gland, and thereby be perpetuated in nature by this host. Accordingly, experiments were undertaken to determine whether rabies virus introduced peripherally would, in fact, invade and multiply in the brown adipose tissue of insectivorous bats. Data compiled from such a study (Sulkin et al., 1959) are summarized in Table I. Two species of bats, the Mexican free-tailed bat (Tadarida b. mexicana), which is a quasi-hibernator, and the little brown bat (Myotis l. lucifugus), a true hibernator, were used in these experiments. A strain of canine rabies virus isolated from the brain of a fatal human case was used. Each bat received an intramuscular injection into the heavy muscle over the chest of approximately 8000 mouse i.c. $LD_{50}$ contained in 0.1 ml.

Although the Mexican free-tailed bat proved to be relatively unsusceptible to experimental rabies infection, virus was demonstrated in the brown fat of 22 per cent of those animals shown to be infected by viral assay in white Swiss mice. The infection in this species was most evident 20 to 40 days after intramuscular inoculation of virus. On the other hand, rabies virus was found to be widely distributed on the little brown bat 9 to 26 days following inoc-

| Bat Species | No. infected* / No. tested | Per cent infected | Virus demonstrated in** | | |
|---|---|---|---|---|---|
| | | | Brown fat | Salivary gland | Brain |
| Tadarida b. mexicana | 104/492 | 21.1 | 22.1 | 35.6 | 87.5 |
| Myotis l. lucifugus | 54/143 | 41.3 | 30.5 | 16.9 | 91.5 |

Table 1. Susceptibility of bats to canine rabies virus (Thompson strain) following intramuscular inoculation. Compiled from: Sulkin, S. E., et al., 1959. Studies on the pathogenesis of rabies in insectivorous bats. I. Role of brown adipose tissue. J. Esp. Med. 110: 369-388. *Virus inoculum approximately 8,000 mouse intracerebral $LD_{50}$. **Per cent positive among animals shown to be infected.

ulation, and virus concentration in some of the tissues approached the level of the stock mouse brain virus suspension used in inoculating these animals. Virus was demonstrated in the brown fat of 30 per cent of the experimentally infected Myotis. These data, together with the recent demonstration of rabies virus in the brown fat tissue in naturally infected insectivorous bats, would support the hypothesis that this tissue provides nutriment for a latent focus of infection (Bell and Moore, 1960; Sulkin, 1962). Studies are in progress to determine the frequency with which virus can be demonstrated, under natural conditions, in the brown adipose tissue of bats netted in different geographic areas at different times of the year.

The mechanism by which brown fat may actually serve as a depot for storage of rabies virus is still under study. A working hypothesis concerns the possibility that at least in hibernating species, rabies virus sequesters in brown adipose tissue in the quiescent host during the period of hibernation and is subsequently activated by the physiological alterations which occur prior to awakening and by emergence into a warmer environment. Studies concerned with the effect of low environmental temperature on the pathogenesis of rabies in insectivorous bats would suggest that this may actually be the case. The remaining portion of this discussion will be limited to those areas which relate to the subject of this conference.

It is clear from previous discussions in this conference that environmental temperature has been shown to have a significant effect on several experimental virus-host systems, both in vivo and in vitro. It is also apparent that the outcome of such experiments depends on the particular virus used and on the host or cell system under study (Sulkin, 1945; Walker and Boring, 1958; Hoggan and Roizman, 1959; Lwoff, 1959).

During the course of studies on the role of Chiroptera as reservoir hosts for viruses in nature, it became evident that these experiments with bats provided an unusual opportunity for determining the effect of temperature on viral multiplication in the intact animal. The bat does not possess a thermoregulatory mechanism for maintaining a constant normal body temperature, but rather, the body temperature of a bat is that of his environmnet, except when he is in the active state of walking or flying (Hock, 1951; Morrison, 1959).

The body temperature of resting bats has been shown to parallel closely that of their environment over a range of 2° C to 30° C, and the metabolic rate of these animals varies directly with their body temperature (Hock, 1958).

Data have been accumulated comparing the course of experimental viral infections in bats held at 5° C or 10° C with groups held at 24° C or 29° C and with groups held at low temperatures for a period of time and then transferred to room temperature (24° C) or 29° C (Sulkin et al., 1960). Animals held in the cold become torpid within hours of being placed at the low temperature, and we have on occasion determined the rectal temperature of a number of cold-adapted bats and found it to be quite close to ambient. On the other hand, we cannot be positive that animals held at 24° C or 29° C maintained constantly body temperatures equivalent to their environment. For a period of time following the handling associated with virus inoculation and transfer to experimental cages, the bats appear excited and are quite active in moving about their cages. It is likely that during this period the animals have body temperatures well above ambient. Within a day or so, however, they become quiet and hang in groups in their cages; they are seldom seen moving about except in the evening when they come down to the floor of the cage to receive the food and water offered daily. There is doubtlessly considerable variation among body temperatures of individual bats at these higher temperatures, depending on their degree of activity. The difficulties inherent in recording individual body temperatures on large numbers of infected bats precludes the possibility of obtaining such data. In this presentation we will be comparing the course of viral infection in animals whose body temperatures we know to be low (ambient temperature 5° C or 10° C) with the course of the infection in animals whose body temperatures we feel must have fluctuated between ambient (24° C or 29° C) and possibly 36° C or higher (Morrison, 1959).

Early in these studies it became apparent that animals collected during the fall or early winter months survive for longer periods of time in the cold than animals obtained in the spring or summer months. Subsequently, these experiments were initiated only in the fall, even though they could only be repeated or extended on a yearly basis. Furthermore, a recent report by Menaker (1962) indicates that the state of hypothermia achieved with summer bats is not

identical with their winter hibernation, and serves to emphasize the necessity for carrying out low temperature studies in bats gathered during the fall months.

Table II summarizes a study of the suppressive effect of simulated hibernation on the susceptibility of little brown bats to two strains of rabies virus. Of those receiving the canine rabies virus (Thompson) and placed in the cold room, infection was demonstrated in only 7.5 per cent of 40 bats studied over 40 days. The infection rate among animals kept at 29° C was 36 per cent, and virus was demonstrated between the ninth and thirtieth day following virus inoculation. It is interesting that virus was demonstrated more frequently in the brown adipose tissue (25.7 per cent) than in the salivary gland (11.4 per cent). The infection rate was even higher among animals held in the cold for two weeks and then transferred to the warm room. Again virus was demonstrated more frequently in the brown fat (30.4 per cent) than in the salivary gland (17.1 per cent). Quantitative data not shown in this tabulation indicate that virus titers in the few animals which developed evidence of infection while in simulated hibernation were $10^{-1.5}$ or less, while titers in various tissues of animals in the warm room ranged from one log unit to as high as $10^{-4.5}$. In the experiment with the rabies virus (59 V13B) recovered from the pooled brown adipose tissue of naturally infected little brown bats, virus was again demonstrated infequently in tissues of animals inoculated and placed directly in the cold room, but remained viable and was activated when animals were transferred to the warm room. Although the infection rates were similar with both rabies virus strains, a careful study of the results revealed differences in the patterns of infection produced by these viruses in active bats and in those awakened following a period in simulated hibernation. The bat rabies virus occurred more frequently in the brown adipose tissue than in either salivary gland or brain. It can be seen that virus was detectable in the interscapular brown fat in 92 per cent of animals shown to develop infection, and in the salivary gland and brain in only 28 per cent and 50 per cent respectively. Rabies infection was demonstrated in 42 per cent of the animals held in the cold for 17 days before transfer to the warm room, and the infection pattern seemed to be affected by the period in simulated hibernation. In these animals virus was demonstrated in the brown fat, salivary gland, and brain of infected animals with about equal frequency.

| Rabies virus strain | Inoculum (mouse i.c. $LD_{50}$) | Environmental temperature C | No. infected / No. tested | Per cent infected | Virus demonstrated in* | | |
|---|---|---|---|---|---|---|---|
| | | | | | Brown fat | Salivary gland | Brain |
| Canine (Thompson) | 8,000 | 5° | 3/40 | 7.5 | (1) | (1) | (2) |
| | | 29° | 35/97 | 36.1 | 25.7 | 11.4 | 91.4 |
| | | 5°→29°** | 23/47 | 48.9 | 30.4 | 17.1 | 91.3 |
| Bat (59VI3B) | 6,000 | 5° | 2/47 | 4.3 | (1) | 0 | (1) |
| | | 29° | 14/28 | 50.0 | 92.0 | 28.5 | 50.0 |
| | | 5°→29°*** | 21/50 | 42.0 | 57.1 | 52.4 | 66.6 |

Table II. Suppressive effect of simulated hibernation on the susceptibility of bats (Myotis l. lucifugus) to rabies virus following intramuscular inoculation. Data adapted from: Sulkin, S. E., et al., 1960. Studies on the pathogenesis of rabies in insectivorous bats. II. Influence of environmental temperature. J. Exp. Med. 112: 595-617. *Per cent positive among animals shown to be infected; figures in parentheses refer to numbers of animals. **Animals held at 5° C for 14 days before transfer to 29° C. ***Animals held at 5° C for 17 days.

suggesting that after a period of latency in a dormant animal, activated virus may reach the salivary gland more rapidly and with greater frequency. In addition, quantitative studies have indicated that the virus concentration in this tissue is greater than in infected animals which have not experienced a period of hibernation. These data are presented graphically in Figures 1 and 2.

The results of these experiments suggest that seasonal fluctuations in environmental temperature may provide not only a mechanism for virus storage in this reservoir host, but may also increase the chances of virus transmission by this host. It is recognized that this concept may only apply for strains of rabies virus which circulate in bat populations.

## ARTHROPOD-BORNE VIRUS INFECTIONS IN BATS

During the course of studies on experimental rabies infection in insectivorous bats, we became interested in determining how these animals would react to experimental infection with other viral agents. A survey of the literature (Sulkin, 1962) indicated that bats have been associated in various ways with a number of disease-producing agents, suggesting that these animals might act as reservoir hosts for many viruses in nature. An area of particular interest to us, from an epidemiological standpoint, concerns the mechanisms involved in the overwintering of the arthropod-borne viruses. Although investigators have long sought to determine the whereabouts of these viruses during the winter months in temperate zones where mosquito vectors do not carry out year-round transmission cycles, this void still remains in our understanding of the complex biological life cycles of these disease-producing agents. Reports of the susceptibility of bats to experimental infection with various arthropod-borne viruses (Ito and Saito, 1952; Corristan, LaMotte and Smith, 1956; LaMotte, 1958) suggested this animal as an additional reservoir host, but provided only limited information as to the course of the infection or the tissues involved. We were prompted, therefore, to pursue studies on experimental arbovirus infection in bats to de-

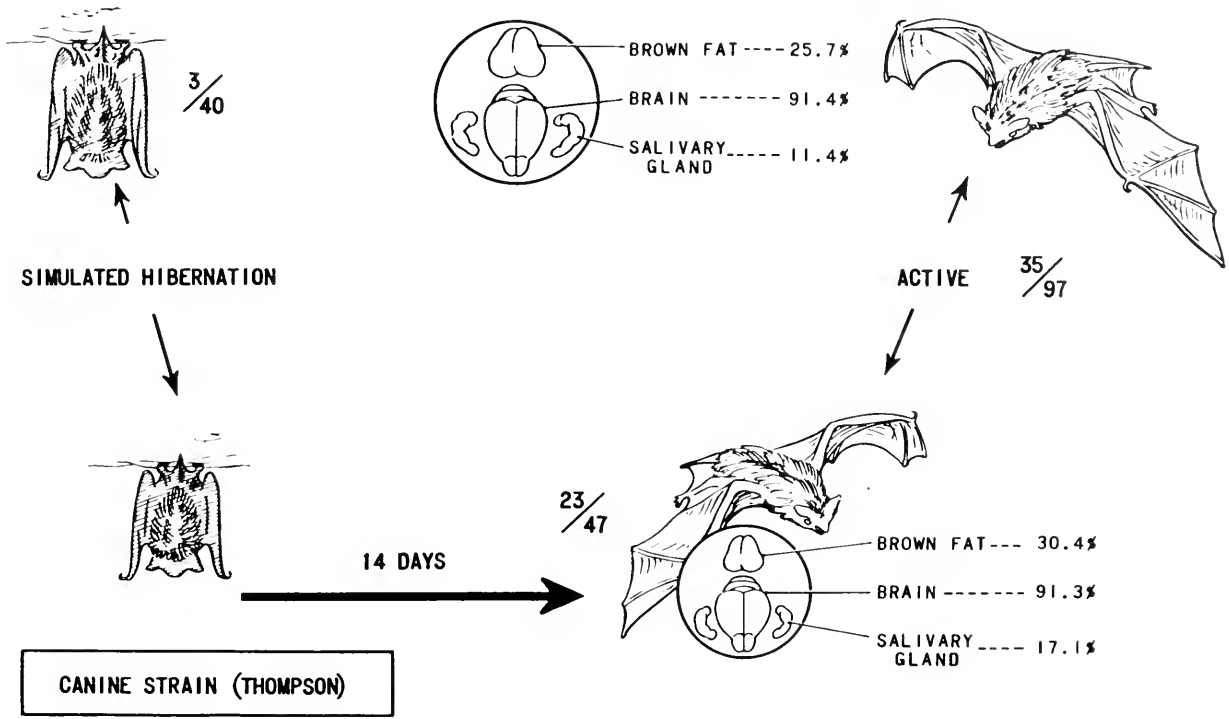


Figure 1. Influence of environmental temperature on frequency of demonstration of canine rabies virus (Thompson strain) in brown fat, brain, and salivary gland of bats (Myotis l. lucifugus) following intramuscular inoculation. Each animal received 8,000 mouse intracerebral $LD_{50}$. Fractions refer to number infected/number tested. Percentages are based on number of animals shown to be infected.

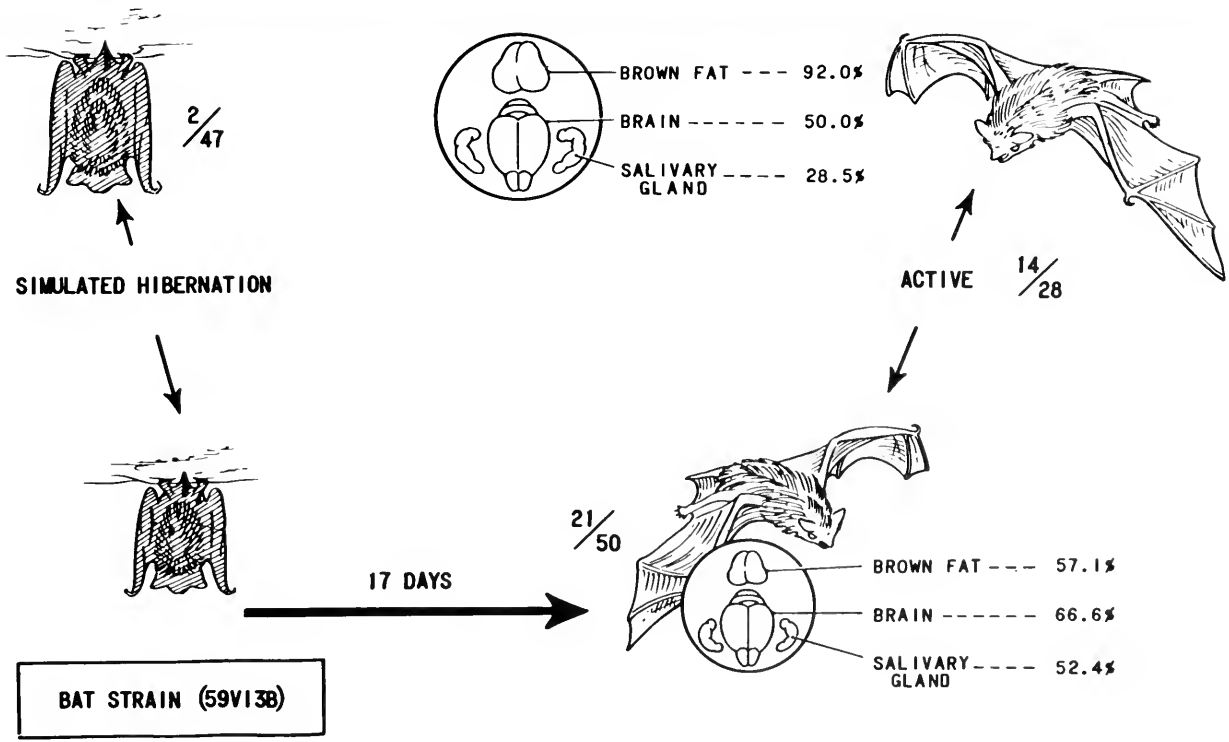


Figure 2. Influence of environmental temperature on frequency of demonstration of bat rabies virus (Strain 59V13B) in brown fat, brain, and salivary gland of bats (Myotis l. lucifugus) following intramuscular inoculation. Each animal received 6,000 mouse intracerebral $LD_{50}$. Fractions refer to number infected/number tested. Percentages are based on number of animals shown to be infected.

determine the degree of susceptibility of various species to certain virus strains and to locate the tissues involved in the infection which provided sites for viral proliferation and resultant viremia. Again, emphasis was placed on determining if virus invaded and multiplied in interscapular brown adipose tissue, thereby providing a mechanism for survival of arbovirus particles in the hibernating animal in a manner similar to that described for rabies virus.

In the initial experiments (Sulkin, Allen and Sims, 1960), we were able to confirm certain aspects of the observations reported by Corristan et al. (1956) and LaMotte (1958). Viremia was demonstrated in bats following peripheral inoculation of Japanese B or St. Louis encephalitis viruses and, in addition, the lipotropic characteristic of these viruses was demonstrated. These experiments have now been extended to include various bat species and several virus strains. Bats were inoculated subcutaneously and placed in specially designed cages which allowed them to receive fresh food and water daily with nimimum danger to the caretakers. The virus inoculum consisted of approximately 150 weanling mouse i.c. $LD_{50}$ of mouse brain suspension, or infected tissue culture fluid, or bat blood. To simulate natural circumstances, bats were kept in near darkness throughout the day and were offered food at the close of each working day. Unless otherwise indicated, animals were maintained at $24^{\circ}$ C $\pm$ $2^{\circ}$ C (relative humidity 65 per cent) and were observed several times daily throughout the course of the experiments for possible signs and symptoms of encephalitis. When tissues were obtained for virus assay, extreme care was taken to rid specimens of as much blood as possible so that resulting virus titers would reflect virus multiplication in a specific tissue and not virus present in blood circulating through that tissue. These experiments, which will be published in detail elsewhere (Sulkin, Allen and Sims, unpublished data), indicate that the Mexican free-tailed bat is only slightly susceptible to the high mouse passage Nakayama strain of Japanese B encephalitis virus, but is significantly more susceptible to a mosquito isolate (OCT-541) which had been through two hamster kidney tissue culture passages and one passage in little brown Myotis. In the latter case, the infection was widely disseminated in animals sacrificed over a period of 3 weeks, with virus concentrations reaching 3 logs or more in blood, brown fat, and kidney. In one instance virus was demonstrated in the brown fat and not in the

blood of an animal sacrificed 21 days after virus inoculation. The passage history of the virus strain seems to determine its degree of infectivity for the Mexican free-tailed bat, where the recently isolated strain is much more infective than the laboratory-adapted strain. Similar results were obtained with two strains of St. Louis encephalitis virus. This bat species is quite resistant to infection with the high mouse passage Hubbard strain, but is quite susceptible to infection with a strain recovered from a flicker bird and in its seventh mouse brain passage. Virus was first demonstrated in blood one to two days after virus inoculation, increased to levels of 4.0 log units by the seventh day, and was still detectable in animals sacrificed about one month post-inoculation. In addition, virus was demonstrated with much greater frequency in brown fat than in brain or kidney. In a few instances virus was demonstrated in tissue preparations from animals that were not viremic at the time of sacrifice.

The little brown bat is highly susceptible to the OCT-541 strain of Japanese B encephalitis virus. Virus was widely distributed in the various tissues assayed during an observation period of about one month; virus was demonstrated in the blood and in brown fat with about equal frequency, and in several instances virus was demonstrated in the brown adipose tissue and not in blood or other tissues tested. This bat species is only slightly susceptible to infection with the flicker bird strain of St. Louis encephalitis virus. The big brown bat (Eptesicus f. fuscus) is highly susceptible to infection with the OCT-541 strain of Japanese B encephalitis virus. All animals developed widespread infection during the first 12 days following virus inoculation, titers in the blood and brown fat reaching more than 3.0 log units in many instances. Subsequent to the twelfth day, the infection seemed gradually to subside; only 2 of 10 animals tested had a detectable viremia by the twenty-fifth day. The susceptibility of this bat species to the other virus strains has not yet been determined.

# INFLUENCE OF TEMPERATURE ON ANTIBODY PRODUCTION IN BATS

In planning studies on the influence of hibernation on the progress of these experimental viral infections in bats, it seemed desirable to determine the pattern of antibody production in these animals following peripheral inoculation of various arthropod-borne viruses. As pointed out earlier, the unique thermoregulatory mechanism of bats sets them apart from other animals and suggests this animal as an ideal experimental host whose temperature and metabolism may be altered at will, permitting the influence of temperature on infection and antibody production to be studied over extended periods. The use of bats also permits the achievement of hypothermia without the use of drugs or surgery which might have an independent effect on the physiochemical balance of the host.

A bacterial antigen was selected for a pilot experiment on the effect of temperature on antibody production because of the ease with which one can demonstrate agglutinating antibodies. The typhoid-H antigen-antibody system was chosen as representing a relatively simple immunization procedure and antibody assay technique requiring very small serum samples, and the big brown bat was used because it can withstand repeated bleeding by cardiac puncture. One group of bats was maintained at room temperature (26° C) and another in simulated hibernation (10° C). Rectal temperatures were taken periodically. All bats received 3 intraperitoneal doses of 0.25 ml each of an 8-hour formalized broth culture of Salmonella typhosa on days 0, 15 and 24 of the experiment. Several animals were bled periodically and all were bled on the 35th day following the initial immunizing dose. The antibody level of each serum sample was determined by means of the tube agglutination test. The results indicate that the ability of the big brown bat to produce antibody in response to injections of a bacterial antigen is dependent upon its body temperature, which parallels that of the environment (Fletcher et al., 1962). After 35 days and 3 doses of antigen, over 70 per cent of the animals had agglutinin titers ranging from 1:8 to 1:672. In striking contrast, the bats held at 10° C failed to produce any detectable antibody during this period. Some animals which had been

maintained at room temperature were transferred to the cold room on the 42nd day, and blood specimens were obtained one month later. Persistence of antibody was observed in 5 bats, but in each instance agglutinin titer dropped. Bats kept in simulated hibernation for 42 days were transferred to room temperature and one month later (or 72 days after initiation of the experiment) blood samples were obtained. Although no additional antigen was administered upon transfer to room temperature, 6 of 10 animals uniformly negative after 30 days in the cold possessed agglutinating antibody one month after transfer to the warm room. Presumably, antigen persisted during hibernation.

Several studies have been made to determine the immune response of big brown bats experimentally infected with Japanese B encephalitis virus and held at room temperature or in simulated hibernation for extended periods. Results indicate an extremely close association between the cessation of viremia and the appearance of detectable neutralizing antibody. Even virus may be detected in low titer in blood samples containing neutralizing antibody. Also, preliminary studies indicate that bats experimentally infected with this virus do not regularly produce antibodies detectable by the complement fixation (CF) and hemagglutination-inhibition (HI) techniques currently in use. Experiments designed to study the antibody response to the hibernating bat and the fate of any neutralizing antibody present in the animals entering hibernation indicate that antibody is produced by bats kept at room temperature, while none is detectable in those held in the cold. As in the case with the bacterial antigen, there is evidence that transfer to room temperature stimulates antibody syntheses. In general, it has been found that bats which had equivocal titers (1.0 to 1.7 LNI)[2] at 17 to 21 days were usually strongly positive about 2 months post-inoculation. Although viremia develops in all experimentally infected bats, some fail to develop even equivocal titers. As was the case in experiments with the bacterial antigen, none of the bats aroduced neutralizing antibody in response to Japanese B encephalitis virus during 43 days at 10° C. Similar results were obtained with some bats which were inoculated with rabies virus and bled after 43 days in the cold (Sulkin et al., 1960).

2 Log neutralization index (LNI). An LNI of < 1.0 was considered negative, 1.0 to 1.7 equivocal, and 1.7 or above positive.

# INFLUENCE OF HIBERNATION ON INFECTION AND ANTIBODY RESPONSE OF BIG BROWN BATS INOCULATED WITH JAPANESE B ENCEPHALITIS VIRUS

Having established the susceptibility of the big brown bat (Eptesicus f. fuscus) to experimental infection with Japanese B encephalitis virus and determined the duration of the viremic phase, the degree of involvement of brown adipose tissue, brain, and kidney, and learned something of the antibody response of infected animals held at room temperature, the influence of low temperature on this infectious process could then be studied. In a study on Japanese B encephalitis infection in bats during simulated hibernation, LaMotte (1958) reported that viremia is suppressed in animals placed at $10^{\circ}$ C immediately after inoculation; upon transfer to room temperature after as long as 3 months in the cold, demonstrable levels of virus appeared in the blood within 2 to 5 days.

In designing our studies, we have attempted to reproduce in the laboratory situations as they might occur in nature. Whenever possible, experiments are planned so that the periods in which animals are held at low temperature in the laboratory correspond to the season of natural hibernation in an effort to simulate hibernation rather than merely attain a state of hypothermia (Menaker, 1962). Groups of bats are placed in cold rooms at varying times following virus incoulation to represent (1) animals entering hibernation immediately after becoming infected and before infection develops to a demonstrable level; (2) animals entering hibernation at the peak of the infectious cycle when virus is present in blood and other tissues; and (3) animals entering hibernation while in the immune phase of the infection when virus is no longer regularly demonstrable in blood or other tissues and significant levels of neutralizing antibody are present. Although these studies are still in progress, we have analyzed sufficient data to enable us to construct a schematic diagram which we believe to be representative of experimental infection with this virus in big brown bats under the various environmental conditions described (Fig. 3).

The uppermost section in Figure 3 depicts infection and antibody

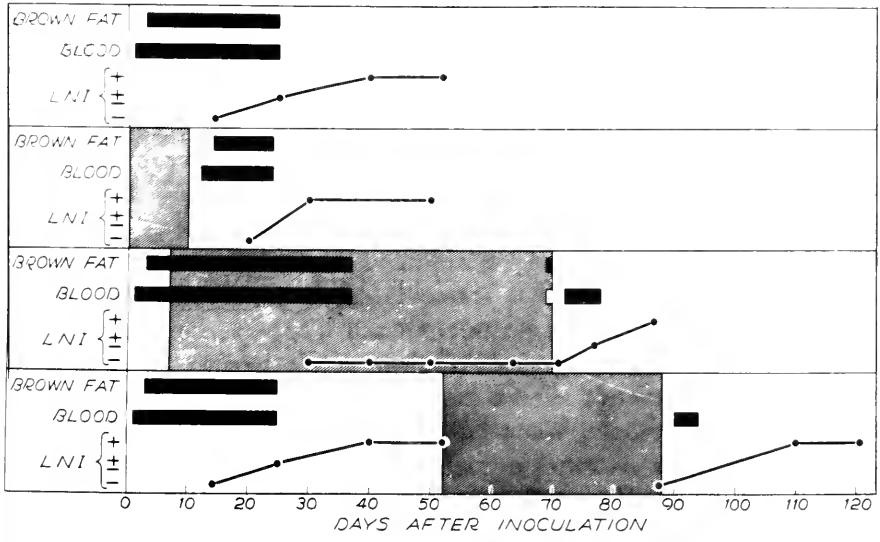


Figure 3. Schematic representation indicating suppressive effect of simulated hibernation on progress of infection and immunologic response of bats (Eptesicus f. fuscus) following intramuscular inoculation of Japanese B encephalitis virus (150 weanling mouse i. c. $LD_{50}$). Shaded areas refer to periods in simulated hibernation (10° C). Solid bars indicate virus demonstrated by intracerebral inoculation of weanling mice. Clear bar indicates no virus recovered. Log neutralization index (LNI) indicated by curves.

response in animals held at room temperature from time of inoculation. Some animals become viremic as early as 24 to 48 hours after receiving 150 $LD_{50}$ doses of virus, whereas virus is seldom isolated from brown adipose tissue before the 3rd or 4th day post-inoculation. We indicate demonstration of virus in blood and brown fat over a period of approximately 25 days. It is difficult to define precisely the duration of active infection, since there appears to be much individual variation. We do know that virus can be demonstrated in brown fat and/or blood of virtually all animals tested 7 to 12 days after receiving virus and that subsequently, the number of infected animals and the concentrations of virus in their tissues gradually diminish. This decrease in viral proliferation coincides with the time when neutralizing antibodies first become detectable, and it is at this point that we have demonstrated on occasion low concentrations of virus in the blood of animals with positive neutralization indices. A significant number of animals have positive LNI 30 to 50 days post-inoculation, although some infected animals never develop antibody detectable by the methods used. Studies on the

persistence of antibody and the occurrence of cyclic episodes of infection in animals held at room temperature are incomplete at this time.

In the remaining sections of the figure, the shaded areas indicate the periods following injection of virus when bats are maintained at low temperature (10° C) and the influence of these periods of simulated hibernation on the progress of infection has been plotted. When animals are placed at 10° C immediately after inoculation, viral multiplication is suppressed. Occasionally, one can demonstrate a trace of virus in blood or brown fat, but it is difficult to determine by assay of specimens in mice the fate of the virus inoculum during this period in the cold. When the animals are transferred to room temperature, virus reaches demonstrable levels in blood and brown fat 2 to 3 days later. It appears that the infection which occurs following a period of suppression in the cold is of shorter duration than in room temperature groups, and antibody reaches significant levels more rapidly. On the other hand, bats placed at 10° C one week after virus inoculation, while at the peak of the infection cycle, seem to suffer prolonged infection. Virus can be isolated regularly from brown fat and blood for at least 30 days after the animals are placed in the cold. Although subsequent data are incomplete, we have included an instance in which virus was isolated from the brown adipose tissue of a bat more than 2 months after the animal was placed in simulated hibernation. The blood of this animal did not contain demonstrable virus, but companion animals circulated virus 3 days after transfer to room temperature. None of the infected bats developed antibody while in the cold, but plasma samples with positive LNI were obtained from bats in this group approximately 2 weeks after transfer to room temperature. In still another series of experiments mapped in the bottom section of Figure 3 we are attempting to determine if an animal entering hibernation in an immune state could, upon arousal, circulate virus again spontaneously. Data along these lines accumulate slowly, since infected bats must be held at room temperature 30 to 50 days for antibody development, then withstand an additional month or more at 10° C and survive transfer back to room temperature before the end results can be obtained. At the present time we have some data indicating that at least some bats with positive LNI, when placed in the cold, lose their antibody during a period of a month or more in hibernation

and upon transfer to room temperature 3 months after the initiation of the original infection may experience a second cycle of infection and antibody response.

## DISCUSSION AND SUMMARY

Our primary interest in studying the influence of low temperature on experimental viral infections in bats has been to accumulate information which would help in understanding how periods of winter hibernation could affect natural virus infections in these animals. As might be expected, the depressed metabolic state and the low body temperature of bats held at $5^{\circ}$ C or $10^{\circ}$ C are not conducive to active viral multiplication. Of far greater interest is the observation that bats in simulated hibernation are able to sustain viral infections during this period of inactivity. Although virus does not appear to replicate at a readily measurable rate during this period, viability is maintained. Upon transfer to a warmer environment simulating spring arousal, virus lying dormant in brown adipose and/or other tissues begins to multiply and active infection, demonstrable by conventional assay methods, ensues. This sustenance of viral particles in the hibernating bat seems more remarkable when accomplished in animals placed at low temperature immediately after virus inoculation. In our studies with rabies virus, animals were inoculated intramuscularly and placed immediately at $5^{\circ}$ C or $10^{\circ}$ C, allowing no time for virus to attach to or penetrate the cells of the animals at room temperature or the higher temperatures believed to be optimum for this animal virus; yet some bats transferred to a warm environment even a month or more later were subsequently shown to develop rabies infection. During the periods inoculated animals were held at low temperature, only an occasional animal could be proved infected upon sacrifice, and in all cases rabies virus was demonstrated in low titer, indicating that virus was not multiplying very rapidly in these bats. Evidence was obtained, however, in an experiment with a bat rabies virus strain in little brown Myotis, that some type of viral activity occurred during the period the inoculated animals were held at $5^{\circ}$ C which resulted in what appears to

be a modification of the virus strain. Sadler and Enright (1959) have recorded the suppressive effect of low temperature on rabies infection in bats and have correlated it with the lowered metabolic rate of the animals. These investigators found no evidence of rabies virus multiplication in animals placed at low temperature immediately after virus inoculation, but if infection were allowed to develop for 6 days at 22° C prior to transfer of animals to 4° C, some evidence was obtained of virus multiplication in the brains of animals which died after 30 to 90 days in the cold.

The experimental data recorded to date on the influence of low temperature on rabies virus infection in bats suggest that winter hibernation may have a profound effect on natural rabies infections in these animals. There is no doubt that the period of winter dormancy could exert a sparing effect perhaps sufficient to maintain a constant state of latency in a large population. In addition, there is the suggestion that rabies virus particles which overwinter in the tissues of the bat and are subject to either complete dormancy or a shift to a much decreased rate of multiplication may be altered by this period at below optimum temperature.

Because the big brown bat is highly susceptible to Japanese B encephalitis virus and suffers an infection which can be characterized as to incubation time, duration of the initial infectious phase, and antibody response, the format of our low temperature studies with this virus-host system is more inclusive than has been possible in work with rabies virus. Assuming that in a given population animals would vary as to the stage of their natural arbovirus infection, we are studying the effects of simulated hibernation on groups of animals which are pre-viremic, viremic or post-viremic, and immune. The results of these studies allow us to speculate along the following lines: (1) bats which enter hibernation soon after receiving an infective dose of Japanese B encephalitis virus, but before infection has developed to demonstrable levels, would be capable of sustaining infection through months of winter hibernation and upon arousal in the spring provide infective blood for feeding vectors; (2) bats which enter hibernation with high titers of Japanese B encephalitis virus in blood and tissues apparently suffer a prolonged infection, possibly due to the suppression of antibody production in the cold; (These animals could provide infective blood for at least a month for mos-

quitoes present at the hibernating site. In this regard, it is of interest that LaMotte (1958) has shown that mosquitoes will feed on bats at 10° C. Thus the viremic, hibernating bat could provide a mid-winter feeding of infected blood for mosquitoes, making possible a two-step winter transmission chain as suggested by Bellamy, Reeves and Scrivani (1958) for western equine encephalitis virus in birds and mosquitoes. In addition, the bat which enters hibernation in a viremic state, although his blood titer gradually decreases during the winter, would be capable of circulating virus again upon arousal in the spring) and (3) bats which enter hibernation at a time when they possess neutralizing antibodies against Japanese B encephalitis virus may show negative neutralization indices by the termination of their winter sleep and the warmer environmental temperature, together with the physiological alterations which occur at time of arousal and emergence, could serve to activate latent Japanese encephalitis virus infection and produce viremic hosts without the necessity of re-infection.

We feel that these studies demonstrating experimentally the various conditions under which the hibernating bat can serve to overwinter an arbovirus add strong supportive evidence to the growing concept of the role of hibernating animals in the maintenance of these agents in nature during periods when vectors are not active. Another hibernating mammal, the hedgehog, has been shown experimentally to circulate Russian spring-summer encephalitis virus during periods in the cold (van Tongeren, 1958) and experimental studies with western equine encephalitis virus in snakes (Thomas and Eklund, 1960) and Japanese B encephalitis in frogs (Chang, 1958) suggest that these poikilothermic animals may be capable of overwintering these viruses.

In addition to the broad epidemiological aspects of the studies presented here, the compiled data also provide information of a more fundamental nature relative to the influence of temperature on the virus-cell-host relationship per se. There is no doubt that the multiplication rate of both rabies and Japanese B encephalitis viruses is suppressed in the bat by maintaining the animal at low temperature. The manner in which infection with both these agents is sustained for long periods of time in the dormant animal, however, suggests that some type of viral activity occurs in this host at low

temperature. The apparent alteration in the bat rabies virus strain which occurred as a result of a period of "incubation" in bats at $5^{\circ}$ C further supports this concept. There is also indication that the Japanese B encephalitis virus may be altered by passage through bats at $10^{\circ}$ C. The virus demonstrable in the blood and other tissues of these animals upon transfer to $24^{\circ}$ C often produces a disease in mice characterized by increased incubation time, bizarre paralytic symptoms and frequently, recovery. In experiments now in progress we are studying strains of viruses following single and multiple passages through bats held at low temperature. These in vivo studies are being paralleled with in vitro experiments using monolayer and explant cultures of bat brown fat, kidney and embryonic tissue, as well as preparations of tissues from warm-blooded animals in an effort to produce virus strains altered by passage at low temperature in different host systems. A study of virus strains altered by the pressures of low temperature exerted in the intact bat, cultures of bat tissues, and cultures of tissues from warm-blooded animals should provide devinitive information concerning the mechanisms of temperature-induced variations in animal viruses.

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## DISCUSSION

CAMPBELL: Did you do electrophoretic patterns of the hibernating bats?

SULKIN: We are doing them now, Dr. Campbell, and we are finding things that I haven't yet had time to sit down and figure out, but we are seeing some very strange things in these electrophoretic patterns. I really don't know what to make of it.

ANDREWES: I understand that viremia is absent in hibernating snakes and then comes back again, as in your bats. When they come out of hibernation, is there any evidence in your bats as to whether this is a function of temperature of not; or hasn't that been done?

SULKIN: I don't think we have enough data. Perhaps Dr. Marcus knows. I don't think so.

SCHMIDT: Many times you used the term "simulated hibernation". Would you care to define it?

SULKIN: I suppose I could delete the word "simulated", but I think that when you take a bat away from his natural environment and then try to simulate these circumstances in the laboratory, there is a difference. I think that when we net the bats in the fall at the time when they ordinarily would be going into hibernation, we are dealing with true hibernation. This is not the case when such experiments are carried out with bats netted in the summer. Such animals, when placed at low temperatures, are hypothermic.[1]

WALKER: You don't see any difference in your experiments, though, in these two seasons?

1 Menaker, J. 1962. Cell and Comp. Physiol. 59: 163-173.

SULKIN: Well, there are many differences in the same bat species, Eptesicus fuscus. You can do an experiment of this sort. You can collect bats in the fall of the year and use thermocouples and record body temperature of these bats. Now, if you take a bat just prior to the time he is goint into hibernation, and then place him at about $8^{o}$ C to $10^{o}$ C in the laboratory, then record temperatures over a period of time, rectal temperature will march along at a very steady state. If you stimulate this bat with a pair of forceps, the temperature goes up, and goes up very promptly. If you take the same species, Eptesicus fuscus, the big brown bat, and you do the same experiment, but do it in July instead using the same probe and the same amount of insertion into the rectum and so on, everything identically the same, then you can stimulate this bat and nothing happens. We have done this with many bats. So this is one significant difference between the true hibernating bat and the simulated hibernating bat.

TRAPANI: Don't they wake up during the winter time for feeding?

SULKIN: No, they stay in the cage.

TRAPANI: In their natural habitat?

SULKIN: In their natural habitat they don't feed; they store food in their brown fat, presumably, and this is enough to keep them going. One can do this with hamsters, too, but it's a trick.

BERRY: In Texas, doesn't the temperature get high enough to bring them out of the hibernating state?

SULKIN: Most of the bats in Texas are not true hibernating species, but migratory species. During mid-winter they migrate south to an area where the temperature is optimum.

BERRY: But bats are in the caves in Texas in the winter time.

SULKIN: Yes, many Mexican free-tailed bats can be found in

certain of the caves during the winter time.

BERRY: But they can't be hibernating all the time, can they?

SULKIN: Most of the bats in Texas, and there are millions and millions of Mexican free-tails there, are not of a true hibernating species. They go out for night time feeding. Consistent with that is the fact that they have very little brown fat in the interscapular region, so it's very difficult to do experiments with this particular species.

BERRY: Just out of curiosity, how do you feed them?

SULKIN: The big brown bats (Eptesicus f. fuscus) are hand-fed meal worms the first week we get them into the laboratory. Most of them learn to feed in about 2 to 3 days. Then we put them on a concoction that we have prepared which consists of cottage cheese, banana, and meal worms, to which is added liver extract with iron and multivitamins.

BERRY: They won't breed?

SULKIN: No. They don't breed under laboratory conditions, but many of the gravid bats have delivered their young in the laboratory.

SCHMIDT: Concerning true hibernation or simulated hibernation in the same species, I believe you indicated that if you induce this simulated hibernation during the summer months, this animal is then under stress and not truly hibernating. I am wondering if he is burning excessive amounts of body tissue of some sort to accommodate himself. Have you made any measurements of weight loss comparing these two periods of time?

SULKIN: No, we haven't done any good experiments along this line. We are so convinced that this is a different host when induced to hibernate during the summertime that we do these experiments on a yearly basis in the late fall.

CAMPBELL: Have you ever injected the brown fat?

SULKIN: Yes, into a non-hibernating animal. The experiments don't work too well. Two sets of experiments have been reported previously, and conflicting results were obtained.[2,3]

BLAIR: Bigelow has done this in Toronto.

SULKIN: These are extracts, and they are not very pure.

BLAIR: He transplanted the brown fat and the results were entirely negative.

SULKIN: Nobody has as yet been able to define the active component in brown fat tissue that is likely to be related to hibernation. And I think that there is a growing interest in this tissue as the true mechanism of hibernation.

TRAPANI: One thing we tried which seemed to indicate some activity of brown fat was this: We made a saline extract out of brown fat obtained from the Guinea pigs exposed to 2° C for about 10 weeks. Ordinarily, you put Guinea pigs into -15° C and they don't do very well; they die quickly. However, when saline extract of brown fat was injected into animals kept at -15° C, they survived another day or so. We never tried it again because of the lack of time.

SULKIN: I think that if you tried it again, it might not work. When saline extracts of brown fat were used by previous investigators, they yielded inconsistant results.

ANDREWES: We have been hearing quite a lot in the last two days on the subject of cold and other stressing factors. There is one form of stress that has never been mentioned. There were some experiments done a good many years ago on pneumococcal infections in partly-immunized mice when it was shown

2 Zirm. 1956. Zschr. f Naturforsch. 11: 530-535.

3 Kross. 1933. Zschr. f. d. ges. Neurol. Psych. 146: 208-218.

that these infections could be activated by the stress of alcohol. This is a subject which needs further investigation.

# MICROBIOLOGICAL ASPECTS OF HIBERNATION IN GROUND SQUIRRELS

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ABSTRACT

Studies were designed to determine the effect of reduced temperatures on the normal bacterial and viral flora of the experimental animals and on the fate of other microorganisms artificially introduced. Determinations were made of the number of coliform bacilli, fecal streptococci, and psychrophilic organisms present in the fecal material before and immediately after hibernation. In addition, the total viable aerobic cell count was determined. All counts were based on the number of organisms per gram (dry weight) of fecal materal. The data indicate a gradual 3 log increase in the number of psychrophiles and a simultaneous decrease of similar magnitude in the number of coliform bacilli during periods of hibernation. No change which could be associated with hibernation was noted in either the total cell count or the number of fecal streptococci. The animals were shown to be free of viruses capable of inducing a cytopathogenic effect in monolayers of either HeLa, human amnion, or monkey kidney cells. Fecal samples from each of 32 animals were repeatedly tested over a period of four months. Bacterial viruses (Escherichia coli phage) have been demonstrated in only one of the 40 ground squirrels examined. Several strains of E. coli isolated from the animals were used as the host cell in the test systems. The effect of hibernation on the presence of artificially introduced ECHO-6 and Coxsackie B-3 viruses and E. coli phage in the intestinal tract of ground squirrels is being evaluated.

Hibernating mammals provide an excellent means for studying the effect of cold on the biology of experimental infection from the standpoint of both the host and the parasite. These animals represent a unique situation in which a normal system is available in the same species, at widely separated temperatures. For instance, in the active state a ground squirrel has a normal body temperature of approximately $37^{\circ}$ C, whereas in hibernation its normal temperature may approach $0^{\circ}$ C.

The studies reported here deal with the effect of hibernation on

certain components of the normal bacterial and viral flora and on the retention of specific viral agents in the intestinal tract of ground squirrels.

Since hibernation is such an important feature of these investigations, a few words about some of its salient characteristics are in order. First of all, it is important to recognize that hibernation is a natural phenomenon for certain animals and that it is quite different from experimental hypothermia. Hibernation is a physiologically controlled and regulated process, while experimental hypothermia is a result of weakened or overcome normal mechanisms of temperature regulation. Chilling occurs in enforced hypothermia in spite of a concerted effort on the part of the animal to maintain its normal temperature. On the contrary, hibernation is a passive, yet deliberate process in which declines in metabolic rate, respiratory rate, and heart rate precede the drop in body temperature.

Within a limited range, the body temperature of the hibernating ground squirrel parallels that of the environment, usually remaining 0.5° C to 3° C above the ambient temperature (Johnson, 1931). Mayer (1960) reported an average rectal temperature of 4.2° C for ground squirrels hibernating in an environmental temperature of 1.6° C. The heart rate and respiratory rate are slowed to about 3 per min, and the metabolic rate is between 1/30 and 1/100 of that found in the active "resting" animal. Vascular changes include a decrease in blood pressure as the animal enters hibernation followed by vasoconstriction once the torpid state has been achieved (Lyman and O'Brien, 1960). There appears to be a pronounced leucopenia during hibernation (Svihla, 1953) which has not been explained. Although the rate is very slow, Brock (1960) reports that there is actually some manufacture of red blood cells during deep hibernation.

There are indications that some of the endocrines play a role in hibernation, although the specific data are conflicting. Most workers agree that an involution of the endocrines takes place before the animal enters hibernation. Indeed, Kayser (1955) insists that hibernation will not take place without this involution.

Although hibernation is part of a yearly cycle of events for ground squirrels, it is not clearly understood what specific factors are im-

portant in determining when they will enter or emerge from the hibernating state. It would appear that the animal receives few, if any, clues from its environment, such as photoperiodicity or temperature (Pengelley and Fischer, 1957).

Ground squirrels and other mammalian hibernators undergo natural awakenings from time to time during the hibernating period (Lyman and Chatfield, 1955). Kayser (1960) reported that the average period of continuous hibernation in ground squirrels is about 21 days. In our work we have been somewhat less successful. The cause of the periodic arousals in undisturbed animals has yet to be explained.

For a more complete survey of the physiology of mammalian hibernation, the reader is referred to the recent publication edited by Lyman and Dawe (1960).

## MATERIALS AND METHODS

Arctic ground squirrels (Spermophilus undulatus), adult animals of both sexes, were captured in the Paxson Lake area of central Alaska during the month of August. They were housed individually in metal cages equipped with removable wire mesh floors and catch pans. Their diet consisted of apples, carrots, and Labena pellets (Ralston Purina Co., St. Louis, Mo.). They were observed in the colony for 2 months before the studies were initiated. Hibernation was induced by placing the animals in a cold room at an ambient temperature of $3^{\circ}$ C $\pm$ $1^{\circ}$ C for the bacterial work and $5^{\circ}$ C $\pm$ $1^{\circ}$ C for the viral studies. These temperatures were maintained throughout each of the respective investigations. The animals were observed daily but not disturbed unnecessarily, because various stimuli may trigger an arousal from the hibernating state.

The primary explants of monkey kidney cells were obtained from Shamrock Farms (Middletown, New York) and maintained on Eagles basal medium with Hank's balanced salt solution (BSS). HeLa monolayers were prepared from the S-3 clone, kindly supplied by

Dr. John L. Riggs of the University of Michigan, using 90 per cent Eagles BM and 10 per cent calf serum as the growth medium. For maintenance, the calf serum was reduced to 5 per cent. The same growth and maintenance media were used for the cultures of the RA strain of human amnion cells.

The specific viral agents used were the D'Amori strain of ECHO-6 virus and the Nancy $m_5K_3$ strain of Coxsackie B-3.

## Bacterial Studies

Investigations were made of the normal intestinal flora of 15 adult ground squirrels both in the active state and following periods of deep hibernation. Fresh fecal droppings were collected on filter paper, and those contaminated with voided urine were excluded. Specimens were kept at $4^{\circ}$ C until processing, which took place within 2 hours after defecation. The droppings from an individual animal were weighed and combined with an equal weight of sterile distilled water. From this a uniform suspension was made with the aid of a sterile glass rod. Part of the material was used for the determination of the per cent moisture and another portion for the preparation of cultures. Triplicate cultures were inoculated from serial 10-fold dilutions ($10^{-3}$ to $10^{-8}$) of the specimens, and the results obtained were calculated as the number of organisms per gram dry weight of fecal material. Quantitative determinations were made of the coliform bacilli, fecal streptococci, and psychrophilic organisms, in addition to a total viable aerobic cell count. The coliform bacilli were cultured on (Difco) eosin methylene blue agar at $37^{\circ}$ C. The plates were seeded with 0.1 ml of the appropriate dilution which was then spread evenly over the surface of the agar with a bent glass rod. The inoculum was dispensed with a sterile 0.1 ml pipette. Using this technique, well-isolated colonies were consistently obtained. All gram negative rods yielding lactose-positive colonies within 48 hours were arbitrarily considered to be coliform bacilli.

For the enumeration of the psychrophiles, (Difco) tryptone glucose extract (TGE) agar was employed. Pour plates were prepared using an inoculum of 1 ml and the incubation was carried out at $2^{\circ}$ C to $3^{\circ}$ C for 14 days. Organisms producing a visible colony within 14

days were termed psychrophiles. In addition, pour plates were made in TGE agar as above, except that these cultures were incubated at 37° C for 48 hours. The results from these latter cultures were referred to as the total count at 37° C. The number of fecal streptococci was determined using (Difco) azide dextrose agar with an inoculum of 1 ml and 37° C incubation temperature. All plate counts were made using a Quebec colony counter and only those plates having between 30 and 300 colonies were included.

## Bacterial Virus Study

In order to gain some information concerning the normal occurrence of bacterial viruses in the animals, 44 adult ground squirrels were examined repeatedly (154 individual samples) for the presence of bacteriophage in their intestinal contents. The bacterial host cells used were strains of Escherichia coli previously isolated from the animals in the group.

The method used for the detection of phage was as follows. Fresh fecal material was collected in a sterile tarred 50 ml centrifuge tube and the weight of the sample determined. A one to 10 dilution (weight in volume) was prepared in sterile nutrient broth and a uniform suspension made with the aid of a sterile glass rod. Following centrifugation at 17,000 X g for 30 min at 5° C the clear supernatant fluid was withdrawn and tested for the presence of virus by two methods. In the first, 1 ml of the fluid and 1 ml of a 3 hr broth culture of the strain of E. coli being tested was added to 5 ml of trypticase soy broth. This suspension was incubated overnight at 37° C and the supernatant fluid therefrom was assayed for the presence of virus according to the following procedure. Serial 10-fold dilutions ($10^{-1}$ to $10^{-6}$) of the fluid were prepared in sterile nutrient broth and 0.1 ml added to a tube containing 2 ml of melted (43° C) trypticase soy agar (0.7 per cent) and 0.1 ml of an 18 hour broth culture of the test strain of E. coli. This was carefully mixed and poured over the surface of a layer of nutrient agar in a petri plate. These cultures were incubated at 37° C and examined for the presence of virus as indicated by the formation of plaques. In the second method the preliminary overnight incubation was eliminated, and the original supernatant fluid was tested for bacterial virus by the plaque method.

These rather serious attempts to isolate phage disclosed that only one ground squirrel out of the 44 tested was excreting a bacterial virus reactive against any of the host strains used. This virus (E. coli phage 42) would effectively lyse our strain 14 of E. coli. Therefore, we had available a working system which had been isolated from the colony, and 43 animals which were naturally free of the agents involved. Accordingly, a study was made of the effect of hibernation on the retention of this bacterial virus following artificial administration in the intestinal tract of the experimental animals.

Ten squirrels were used in this investigation. Of these, 5 were in deep hibernation and 5 were active but had been in the cold room for the same length of time as those in the hibernating state. The temperature in the hibernaculum was $5^{\circ}$ C, as mentioned earlier. Each animal was given a dose of 1 ml containing $29 \times 10^{9}$ plaque forming units of virus. The virus suspension was cooled to $5^{\circ}$ C and introduced into the stomach by means of a 2 ml syringe fitted with a 2 in. length of polyethylene tubing. The tubing was easily directed down the throat of the animals while they were hibernating. Ether anesthesia was used in the case of the active animals.

Following administration of the virus, quantitative determinations were made for excreted virus by collecting fresh fecal material and examining it by the direct plaque method described above. After each specimen was obtained, the animal was transferred to a clean cage, in an effort to preclude contamination of subsequent specimens.

## Enteric Virus Study

Following these investigations, attempts were made to determine the natural occurrence of enteric viruses in the animals. Thirty-two squirrels were examined repeatedly over a period of 4 months for the presence of enteric viruses in their intestinal contents. It was surprising (justified or not) to find that all were free of viral agents capable of inducing a cytopathogenic effect in monolayers of either HeLa, human amnion, or monkey kidney cells. Therefore, we initiated studies to determine the effect of hibernation on the retention of specific enteric viruses in the intestinal tract of these animals following artificial administration. Preliminary tests had indi-

cated that monkey kidney cells were susceptible to ECHO-6 virus and that Coxsackie B-3 virus could be isolated and propagated in monolayers of HeLa cells. In addition, both viruses could be successfully recovered from the fecal material of animals which had been fed a suspension of the specific agents.

Accordingly, 9 squirrels were given a suspension of $1 \times 10^8$ ECHO-6 virus particles and 8 others were fed $1 \times 10^7$ particles of Coxsackie B-3 virus, using the method of administration described above. Approximately half of the animals in each group were hibernating and half were in the active state; all had been in the cold room for the same period of time. Thereafter, fresh fecal samples were collected at appropriate intervals and tested for the presence of virus using monolayers of monkey kidney and HeLa cells. Serial 10-fold dilutions ($10^{-1}$ to $10^{-8}$) of the fecal material were prepared in Earles BSS and a duplicate series of tubes was inoculated with 0.1 ml of the proper dilutions. Incubation was carried out at $37^{\circ}$ C, and the cultures were examined daily for evidence of a cytopathogenic effect.

## RESULTS AND DISCUSSION

The data indicated a significant increase in the number of psychrophiles and a simultaneous decrease in the number of coliform bacilli following periods of hibernation. On the other hand, very little fluctuation was observed in either the total counts at $37^{\circ}$ C or the numbers of fecal streptococci, and these did not appear to be influenced in any way by hibernation. The values shown represent the averages obtained with the 15 ground squirrels studies. Although there was some variation in the hibernating habits, activity, food consumption, and normal intestinal flora of the individual animals, each of the squirrels tested demonstrated these characteristic trends.

A representative number of the coliform bacilli and psychrophiles were isolated and their temperature-growth relations deter-

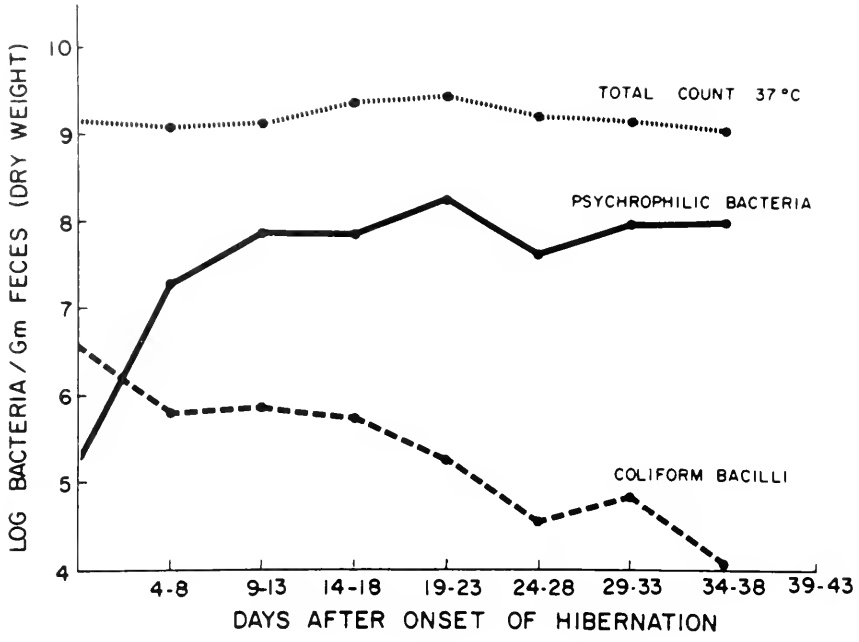


Figure 1. Temperature-growth relations between a representative number of psychrophilic bacteria and coliform bacilli.

mined. None of the coliform bacilli tested grew at temperatures below 5° C. This would imply that little or no multiplication of these organisms occurred in the intestinal tract of the animals while they were in the hibernating state and it would be consistent with the decrease in the counts as shown in Figure 1. The psychrophiles, on the other had, grew well when incubated at temperatures down to 0° C. Although Ingraham and Stokes (1959) report that some psychrophilic bacteria have a maximum growth temperature above 37° C, none of the psychrophiles isolated in our study gave evidence of growth at temperatures above 35° C. During this study the average duration of continuous hibernation was 6 days with extremes of 2 and 19 days. Between periods of hibernation the animals were active for an average of 1.3 days, indicating that they were in hibernation approximately 82 per cent of the time. This figure must be considered conservative, because it takes an active animal from 8 hours to several days to become completely dormant, and the waking process requires about 3 hours (Lyman and Chatfield, 1955).

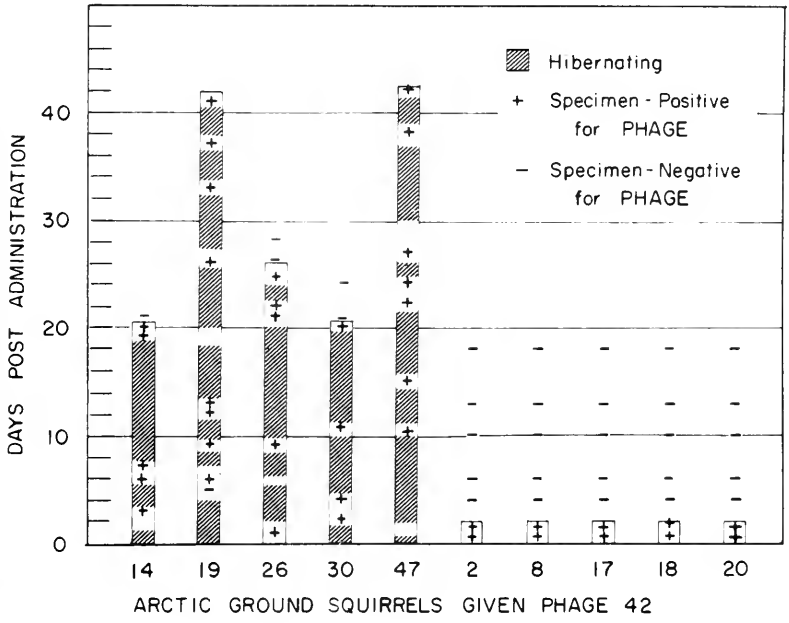


Figure 2. Arctic ground squirrels given phage 42.

The results of the bacterial virus study are presented in Figure 2. In can be seen that the virus was rapidly eliminated from the intestinal tract of the active animals, but was retained for a considerable period of time by the animals which hibernated. It should be noted that each of the hibernating individuals was in an aroused, or active state for periods of time more than that required for complete elimination of the virus in the non-hibernating squirrels. This might be explained by the fact that when the animals come out of hibernation for these short periods, they usually do not eat, even though food may be available. This would, of course, result in a less rapid turn-over of the intestinal contents and thus permit a retention of the virus. In any case, it is not suggested that the extended period of virus retention observed in the hibernating animals was the result of any specific effect, but rather a reflection of the fact that under these conditions it simply takes longer for material to pass through the gastrointestinal tract of the animal. It is likely that the phage is handled as an inert particle, so far as the animal is concerned.

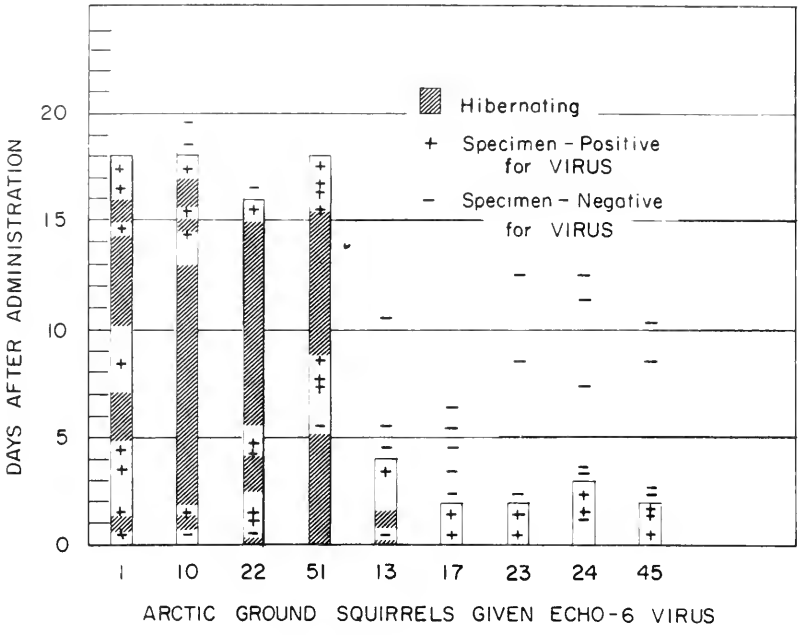


Figure 3. Arctic ground squirrels given Echo-6 virus.

The results of the ECHO-6 studies are shown in Figure 3. As in the case of the bacteriophage, it can be seen that the virus soon disappeared from the intestinal tract of the active animals but could be recovered from the hibernators up to 18 days after administration. Two of the squirrels were still excreting virus when the last sample was collected.

Essentially the same effect was observed in the case of the Coxsackie B-3 virus, as shown in Figure 4. The period of retention in the active animals was somewhat longer than that observed for the bacteriophage and ECHO-6 virus, but these differences may be more apparent than real.

As to the fate of the virus introduced, none of our data gave any evidence that viral replication had occurred. Indeed, we recovered considerably less virus than we administered.

Dempster et al. (1961) report that Coxsackie B-3 virus is in-

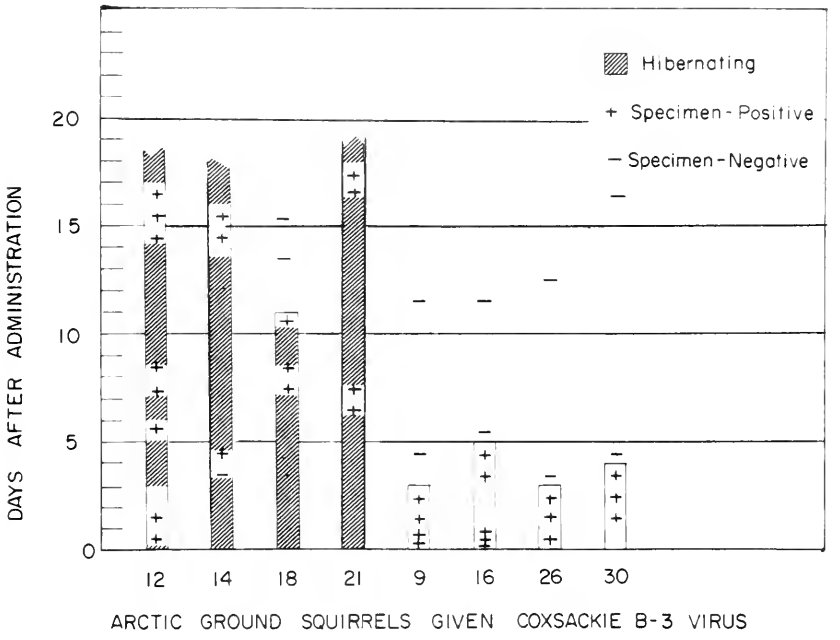


Figure 4. Arctic ground squirrels given Coxsackie B-3 virus.

fectious for ground squirrels in both the hibernating and non-hibernating state. They were able to recover virus up to 50 days, after subcutaneous inoculation, from the brown fat, brain, and heart tissue of animals which had hibernated. On the other hand, their active animals were positive for virus at 4 but not at 14 days after a similar inoculation. They make no mention of the squirrels arousing from hibernation following the injections nor subsequently during the study period and would seem to infer that they observed continuous hibernation for as many as 50 days. Based on our own experience and the published reports of others (Lyman and Chatfield, 1955; Kayser, 1960), periods of continuous hibernation beyond 30 days are unusual; the record, to my knowledge, has been 40 days (Kayser, 1960). We have not as yet examined any tissues from our animals for the presence of virus but expect to do so in some future studies. It is my belief that active virus could remain in the animal so long as hibernation continued. Such findings would, of course, have implications with respect to the possibility of overwintering of enteric and other viral agents.

The resistance of hibernating animals to infection has been investigated by Kalabukhov (1958) and others. The available evidence indicates that resistance to infection is increased during the period of preparation for hibernation as well as during hibernation. There is general agreement that the enhanced resistance is simply a reflection of the physiological state of the animal. Since the infectious agents tested are not capable of reproduction at temperatures found in the hibernating host, one would not expect to find evidence of an active infection under such conditions. At the same time, one might expect very little phagocytic activity at such reduced temperatures, and it is known that a leucopenia occurs during hibernation (Svihla, 1953). These observations would suggest that increased resistance in the hibernating state is probably not related to the cellular mechanisms of resistance.

Adequate data are not available concerning the humoral resistance of hibernators. Jaroslow and Smith (1961) have studied the disappearance of antigen from the circulatory system of hibernating ground squirrels. Their data indicate that there is little or no disappearance of homologus or heterologus proteins from the circulation during periods of hibernation. Studies concerning the actual production of antibody during hibernation are lacking.

In our bacterial studies we have shown that the psychrophiles grow well at temperatures down to $0^{\circ}$ C, and that they increase considerably in number in the intestinal tract during hibernation. Most of the psychrophilic organisms we have encountered have been gram negative rods and producers of endotoxin. It is conceivable that with the animal's normal mechanisms of resistance either inoperative or functioning with reduced efficiency, these organisms might find their way out of the intestinal tract and into other parts of the body such as the blood stream. It is tempting for me to speculate that one of Nature's reasons for programming those unexplained spontaneous arousals, which are accomplished at great expense of energy to the animal, might be to clear the circulation of these and other invading toxic elements. One of the things we plan to do in the future is to check the blood of the hibernating squirrels for psychrophiles and other endogenous microorganisms.

## SUMMARY

The effect of reduced temperatures (associated with hibernation) on the normal bacterial flora and on the fate of specific viral agents which were artificially introduced into ground squirrels has been studied. The data indicate that a gradual increase in the number of psychrophiles and a simultaneous decrease in the number of coliform bacilli occurs during hibernation. No change, which could be associated with hibernation, was noted in either the total cell count or the number of fecal streptococci.

The viral studies demonstrated that hibernation appears to extend the period of time during which the specific agents tested could be recovered from animals following artificial administration.

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## DISCUSSION

REINHARD: How much fecal material and ingesta remains in the gastrointestinal tract of these hibernating animals?

SCHMIDT: Generally the stomach is empty. This is following prolonged periods, and as I mentioned, they do not eat, usually, during the periods of arousal. This has been investigated by certain workers from the histological standpoint, and so on. Their stomachs are usually empty, although there will be fecal material remaining in the intestinal tract, and they do defecate when they wake up.

REINHARD: Is this a considerable amount throughout the intestinal tract?

SCHMIDT: I don't know what you would call considerable. Our samples are usually about 1 gm.

REINHARD: Has anybody investigated bacterial activity in the intestine in relation to the possible production of materials which could serve as sources of energy to the host, when absorbed?

SCHMIDT: No, as far as I know, but I think the source of energy is the brown fat.

BERRY: Does the total amount of fecal material discharged by the control animals that get rid of the virus in three or four days equal the total amount of fecal material of the hibernating animals which require eighteen days to eliminate the virus?

SCHMIDT: I am not sure I understand you.

BERRY: This would give some measure of digestive tract motility.

SCHMIDT: Yes, it is my feeling that this virus is taking a

ride; in the active animals, it simply gets there quicker.

BERRY: It should be possible to measure the total fecal material discharged.

SCHMIDT: That is right. We have records on this, but I did not think that the data that I had suggested anything very unusual about this, and as I mentioned, I feel that this retention is a mechanical sort of thing. Now, perhaps not in the case of Coxsackie B-3. Dempster[1] has reported active infection in his animals. He's worked with bats and also with ground squirrels of a different species than mine. There are some things that we have to get straightened out about Dempster's work, but he claims that, for instance, the virus actually multiplies during hibernation.

CAMPBELL: What happens if you remove the brown fat before or during hibernation?

SULKIN: I think it would be impossible to do this, because although brown fat is located largely in the interscapular area, it is distributed elsewhere in the body, so you could never do a definitive experiment of this sort.

CAMPBELL: But it is fairly localized in mammals; you could take most of it out.

SULKIN: You could take a pretty good chunk of it out. But there is a lot of brown fat along the spinal column and elsewhere, and it may not take much to do the trick.

WALKER: How important is temperature to this natural hibernation that you speak of? How low must this be in either bats or squirrels?

SCHMIDT: These animals will become groggy, sleepy, torpid and in a state of hibernation around 19° C, for instance, but

1 Dempster et al. 1961. Canad. J. Microbiol. 7: 587-594.

they are like a bear; that is, they are easily aroused. Their body temperature can actually be lower than the ambient temperature. This is supposed to be due to the evaporative cooling effect, but they will enter what is called a state of hibernation at around 19° C, and as you lower the temperature, within reason now, they get in an increasingly deeper state of hibernation. We were trying to get pretty deep hibernation.

TRAPANI: How low is the ambient temperature of the burrow in the natural habitat of the animal?

SCHMIDT: This has been studied by Dr. Mayer[2] here in Alaska. He has rigged up a very clever harness for the animal made out of guaze, on which he attaches a thermocouple. The animal goes down into the burrow and drags the thermocouple with him. He has made recordings throughout the winter. It will actuallv get below 0° C for certain periods of time. I am sure there is a microclimate involved, too, but the temperature is quite steady in the burrow.

TRAPANI: Then they will waken, shiver, and then go back to sleep?

SCHMIDT: The feeling is that they do arouse even in the natural state, periodically, in their burrows, and they will fool around a little while and then go back into hibernation.

ANDREWES: Two questions I'd like to ask. Am I correct in supposing that what you told us implies that these various phages and other viruses that you put into the hibernating animal do persist for very long periods of time in contrast with E. coli which are not only relatively, but actually, disappearing?

SCHMIDT: The E. coli get down to a rather low level. The studies with bacteria were for longer periods of time; however, I think there is a difference. I don't know just where these E. coli go. I can't account for this; there are a number of pe-

2 Mayer, W. V. 1960. Bull. Museum Comp. Zool. Harvard Univ. 124: 131-154.

culiar things here in the bacterial picture. For instance, we see a rather rapid increase in the psychrophiles beyond what you can get in a test tube. You know how slowly psychrophilic organisms grow at, say, 4° C. It takes several days to get much of an increase, but in the squirrel, after he hibernates for a couple of days you find approximately a six log increase and, at the same time, you may observe perhaps a three log decrease in the count of E. coli. I have no explanation for this whatsoever.

WALKER: Could this be a matter of competition in the intestinal tract? Could the conditions be such that one has a greater advantage than the other and E. coli is crowded out?

ANDREWES: I don't see why E. coli should actually die.

SCHMIDT: They won't reproduce at these low temperatures. They have several things working against them, whereas the psychrophiles have everything working their way, as it were. This is the temperature at which the psychorphiles can reproduce, and at the same time, body defenses are rather immobilized so that they can grow unabated.

WALKER: Yes, the pH may change and there are all sorts of things to which E. coli may be susceptible.

SCHMIDT: Well, I have tested for antibiosis by the plate method. It was not an exhaustive study, and although I haven't seen any evidence of this as yet, it is one of the things that certainly should be considered. Also the moisture content of the intestinal contents changes somewhat. In addition, there is a lot of mucus secreted into the intestine, which probably serves to protect the lining during these periods, and this may have an effect on the bacteria and also the virus.

ANDREWES: My second question refers to Dempster's story about the enterovirus multiplying. It would seem from what you have told us that one might infer that this virus is growing at a temperature lower than any other viruses do grow, so one might suspect that any growth there is might occur during these

periods of awakening.

SCHMIDT: That is right. This would seem to be the logical explanation, although Dempster reports viral replication with hibernating bats. In connection with this, I asked Dr. Sulkin about his bats; that is, whether they spontaneously arouse. You see, they're quite a different animal in that they really don't have a thermoregulatory mechanism. But ground squirrels do. You can put ground squirrels in a cold room and if they are ready to hibernate, they will. If they are not, you can keep them there for a long period of time and they won't hibernate. Dempster makes the point that these bats which he kept at $2^{o}$ C did hibernate, and there was virus multiplication. Coxsackie B-3 was the agent he was using. In all fairness to Dempster, let me briefly tell you what he has done. He has made quantitative estimations of virus in squirrels by inoculating suckling mice with dilutions of homogenates of various tissues. With the bats, on the other hand, he reports only "isolation" or "no isolation". He was able to recover the virus from the blood and certain other tissues. I think that he considers viremia as being indicative of viral replication. If we would discuss this with him, which I have not done yet, we might be able to iron this out, but certainly there is this possibility that this minor replication has occurred during these periods of arousal.

NORTHEY: I am sorry to dig up a dead body here, but something is bothering me very much. Was it the consensus of this group that protein metabolism resulting from an increase in total food consumption directly affected antibody production? I think this is a very fundamental point here. In other words, can we say then that the cure for pheumococci pneumonia would be an increase in food intake?

CAMPBELL: I thought I tried to bring this point out when I was talking. Very definitely there are two things involved. Mostly, synthesis of antibody involves a breakdown of the antigen. As I mentioned a while ago, Dixon has pointed out the possibility that under stress of hyperimmunization, the body uses gamma globulin. This is the source of amino acids. Under the normal state it uses free amino acids, so metabolism must play a very

important role in all this type of stress mechanism, if it goes more than three or four days.

NORTHEY: Does it depend upon the antigenicity of the material? This would depend on how antigenetic the material is, in which case it would be independent of the protein metabolism.

CAMPBELL: I don't know what you mean by antigenicity. Some of the things like hemocyanin seem to stimulate more precipitating antibodies than, say, serum albumin. Now, what the basis of this is, I don't know. Some people say that antigenicity is related to molecular weight because all albumin is a very good antigen. But more important is seeing how a material is localized and handled by intracellular enzymes.

# COLD THERAPY IN BACTEREMIC SHOCK

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ABSTRACT

Approximately 10-12 per cent of bacteremic patients go into shock. Mortality is 60-70 per cent despite aggressive therapy, including bacteria sensitive antibiosis. The basic problem in all forms of shock appears to be a disparity between $MRO_2$ and $CDO_2$ (circulatory delivery of oxygen to the cell). The rationale of hypothermia is directed primarily at $MRO_2$ and secondarily at $CDO_2$. At 32° C, $MRO_2$ is one-third of normal, cold pressor effect elevates and sustains arterial blood pressure, heart rate is slowed, and the renal and the CNS flow and ventilation are augmented. The patient is brought into a metabolic environment more commensurate with the reduced perfusion. No direct effect on the bacterial organism or on antibiosis was observed. Similarly, leucocytic response was not altered. Patients were cooled only when they had become refractory to therapy and were in dire straights. Salvage rate was 50 per cent in 52 cooled. Excluding deaths from various causes, 14 (27 per cent) died of unremitting septic shock. In two of these, opsonin indices were found to reduce markedly. Current studies in dogs with no therapy appear to confirm the view that hypothermia (32° C) exerts no direct effect on the organism or on host mechanisms. Death is likely due in part to crippling of the RES.

The syndrome of bacteremic shock has been long recognized as a serious clinical problem (Laennec, 1831). While the most common offending micro-organisms belong to the coliform group, this usually fatal malady can be precipitated by gram positive bacteria, by Rickettsiae and by viruses (Spink, 1960; Ebert and Abernathy, 1961). The pathophysiology is generally pictured as vascular failure or collapse (Romberg, 1899; Gilbert, 1960) and believed due directly or indirectly to endo- or exotoxins (Spink, 1960; Thal and Egner, 1956; Aub et al., 1947). The physiologic dysfunction pattern is similar with both toxins. It is estimated that about 15 per cent of clinical bacteremias develop hypotension and the shock syndrome (Ebert and Abernathy, 1961). Antibiosis along with supportive therapy reduced a 100

per cent mortality to 60 per cent -- 75 per cent in coliform or staphylococcus shock (Altmeier and Cole, 1958; Smith and Vickers, 1960). Ps. aeroguinosa continues to claim a 100 per cent death rate (Moncrief).

Failure to control satisfactorily the staggering losses prompted exploration for help in another direction. While the complex pattern from infection to shock and death is yet to evolve, the underlying problem appears to be hypoxemia. Cellular metabolic requirements ($MRO_2$) in febrile states accelerate. This calls for a proportionate increase in circulation to assure adequate delivery of oxygen ($CDO_2$). At any given metabolic level, the ratio of $CDO_2$ to $MRO_2$ determines the presence or absence of hypoxemia and its degree. As long as the circulation meets the requirements, there is no hypoxemia. In the case of septic shock characterized by circulatory failure, the reduced perfusion results in a proportionate decrease in tissue oxygen tension. The disparity lies with $CDO_2$ (assuming alveolar oxygen tension is maintained). When the ratio falls below a critical level for a given metabolic state, the cell dies. Current treatment is aimed at $CDO_2$ in the form of transfusions, oxygen inhalation and vasopressors. This line of attack is inadequate as indicated by the high rate of death. The next obvious step is to attempt modification of $MRO_2$ in order to bring it more in line with the reduced $CDO_2$ and restore the normal ratio. Hypothermia is a means of doing this. With progressive lowering of core temperature of the homeotherm, there is an exponential fall in oxygen uptake in the absence of shivering (Spurr et al., 1954). At 32° C uptake is reduced by one-third, at 30° C by one-half, at 25° C two-thirds, and four-fifths at 20° C (Blair, 1960). Reduction in blood flow is roughly proportional to that in oxygen consumption. Thus, in septic shock with an already diminished flow, it appeared possible that with a lowering of $MRO_2$ by hypothermia, a more favorable metabolic environment may supervene.

In addition to the significant decrease in metabolism at 32° C, other features at this level provide additional physiological benefits. Cold pressor effect restores and sustains the arterial blood pressure. The heart rate is slowed and its work allayed; the central nervous system is stimulated and reflex mechanisms are augmented; ventilation is improved, and renal flow is enhanced. This has been termed the augmented level (Blair, 1960). The thesis for hypothermia

then, is to modify the metabolic environment of the host with likely no specific action at any given site or enzyme system. In instances such as bacteremic shock, hypothermia would place the metabolic community at a physiologic level more commensurate with the existing attenuated blood flow. Hypothermia as a "therapeutic" tool is quite ancient (Currie, 1798). In recent years a number of reports have attested to the value of hypothermia in septic problems (Blair et al., 1961; Cockett and Goodwin, 1961; Martin, 1958; Drescher, 1960).

This report consists of a description of clinical experiences and some aspects of experimental studies. Evaluation of the influence of hypothermia rested on refractoriness to standard therapy. The patients continued to receive intensive drug and antibiotic treatment while under hypothermia. In order to evaluate the effects of hypothermia per se, bacteremic shock was induced in dogs, one series of which received no treatment other than hypothermia.

## CLINICAL STUDY

### Criteria for Cooling

The sequence of progressive deterioration from an acute infection to septicemia, hypotension, and shock does not adhere to a specific clinical pattern. Too many important factors influence the probabilities of shock in septicemia. Some of these are host resistance, including age and nutrition; type and virulence of offending microorganism; and type, intensity, and timing of treatment. The criteria for the development of shock secondary to sepsis are largely based upon hypotension, tachycardia, hyperpnea, and oliguria. All patients in septic shock were begun on the accepted regimen as enumerated previously. Only those who subsequently became unresponsive and seemed about to die were selected as candidates for hypothermia.

Characteristics of a typical clinical picture are listed in Figure 1. The patient who appeared to be doing fairly well was usually elderly. The temperature dropped slightly, ventilation became irregular, and

1. Elderly debilitated patient
2. Gram negative coliform bacteria
3. Temperature 39° C - 40° C
4. Sudden collapse:
   a. pallor or cyanosis
   b. disturbed sensorium
   c. blood pressure below 70 mm.Hg.
   d. weak, rapid pulse
   e. oliguria
   f. ventilatory disturbance
5. Blood status:
   a. elevated BUN
   b. whole blood deficit
   c. electrolyte deficit
   d. leucopenia often
   e. metabolic acidosis
6. Mortality 65-70 per cent

Figure 1. Clinical features of bacteremic shock.

| Level: | 33° C - 32° C | | | | 35 Patients |
|---|---|---|---|---|---|
| | 37° C | | | | 10 Patients |
| Duration: | 1 | 3 | 7 | 14 | 30 Days |
| | 10 | 16 | 12 | 6 | 1 Day |

Figure 2. Duration of Hypothermia in bacteremic shock.

| Total cases - 45 | | Died - 22 | | | Rate - 50% |
|---|---|---|---|---|---|
| 21-30 | 21-30 | 41-50 | 51-60 | 61-70 | 71+ |
| 5/2 | 3/0 | 3/1 | 15/7 | 14/8 | 5/4 |
| | >50 | | | <50 | |
| | 11/3-27% | | | 34/19-56% | |

Contributory Causes

| | | | |
|---|---|---|---|
| Hypothermia below 30° C | 2 | Electrolyte deficit | 4 |
| Bleeding | 5 | Aspiration | 1 |
| | Unremitting septic shock 10 | | |

Figure 3. Mortality factors.

a pallor engulfed the rapidly deteriorating patient. Urine flow ceased, leucopenia and metabolic derangements were observed often. Refractoriness to therapy, characterized by an almost total breakdown of physiologic compensation, was demonstrated by marked disturbances in sensorium or coma, thready or absent pulse, an arterial blood pressure below 60 mm Hg or unobtainable, and Cheyne-Stokes or Kaussmal breathing. A surface cooling technic was applied utilizing a rubber blanket through which refrigerant fluid (ethyl alcohol) was circulated. The cooling process was stopped at 34° C to 33° C and the patient allowed to drift to a 32° C to 31° C level at which

| Observation | Patients | Per cent |
|---|---|---|
| Hypoglycemia | 4 | 9 |
| Pressor effect | 36 | 80 |
| Improved sensorium | 41 | 91 |
| Pulse slow | 43 | 86 |
| Improved ventilation | 42 | 93 |

Figure 4. Response of septic shock patients to cooling.

| | |
|---|---|
| Pneumonia | 0 |
| Cold Burns | 0 |
| Palsies | 0 |
| Leucopenia | 0 |
| Thrombocytopenia | 0 |
| Drift | Often (2 deaths) |
| Hypoglycemia | 4 |

Figure 5. Complications from hypothermia.

he was then stabilized. By controlling blanket temperature, patients were maintained at 33° C to 32° C for extended periods.

## Results of Clinical Study

A total of 45 patients were cooled for periods of one to thirty days with the majority for a week or less (Fig. 2). Gram negative organisms were responsible for the sepsis in 43 of which E. coli was most commonly predominant. The others were S. aureus, coagulase positive. Positive blood cultures were obtainable in less than 50 per cent. Positive cultures were present during hypothermia and upon rewarming in those who survived.

The salvage rate was 50 per cent (Fig. 3). Only one-half of the deaths were due to unremitting septic shock. At the time of all fatalities antibiosis was specifically bacterio-sensitive. The elderly debilitated patient tolerated septic shock most poorly. Survival rate was 73 per cent in those under 50 years of age and only 44 per cent in those over 50. Two deaths were associated with deeper cooling (28° C and 27° C) and were in individuals over 50 years of age. Response to cooling was uniformly favorable, attesting to the augmenting influence of the level of cooling employed (Fig. 4). However, this response proved no indicator of the outcome. Most of the deaths occurred within 72 hours of cooling. Therefore, survival beyond this time increased probabilities of recovery, unless a complication unrelated to the sepsis intervened. Complications of hypothermia proved to be of little or no consequence where adherence to 32° C was observed (Fig. 5). Four patients developed hypoglycemia, which was adequately managed by glucose infusion.

The criteria for rewarming were based entirely on the subjective course of the hypothermic patient and did not bear necessarily any relationship to the state of the sepsis or host antibacterial response. The primary indication for rewarming was the patient's vocal and muscular objection to the cold state. The sensorium was clear, the arterial blood pressure and pulse stabilized, and ventilation was normal. Elevation in temperature occurred in all patients after rewarming. Hypothermia was reinstituted only when this was accompanied by evidences of shock.

An example of a survivor cooled for eight days is illustrated in Figure 6. This was an 18 year old white female, who developed peritonitis following an appendectomy. Septicemia was evidenced by an elevated temperature of 39° C to 40° C, tachycardia, hyperpnea, and leucopenia. Bacteroides was cultured from the blood stream. Surgical exploration was followed by shock. Failure of response to therapy highlighted by lethargy and coma prompted resort to hypothermia. Within 72 hours there was considerable improvement in the patient's status. Blood cultures remained positive during the hypothermic state and became negative 12 days after hypothermia was discontinued. Rewarming was started on the fourth day, but a convulsive seizure

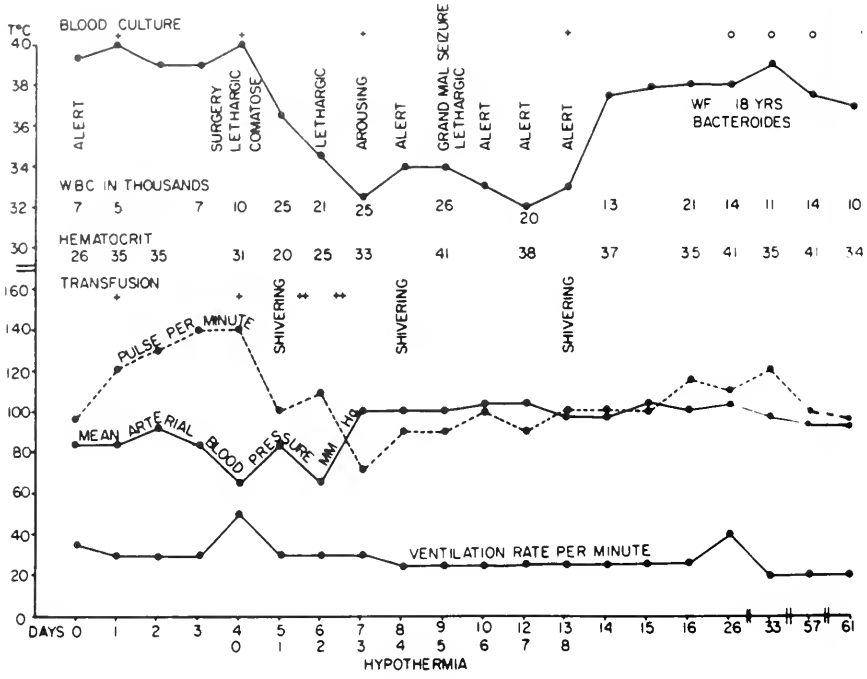


Figure 6. Case of bacteroides septic shock managed with hypothermia.

and coma believed due to brain abscess, necessitated further cooling. Within 24 hours the patient was again awake. The patient complained of the cold and was shivering actively. Rewarming was started again on the eighth day and was followed by an elevated temperature of 38° C. Since the patient's hemodynamics and neurologic status remained normal, no further cooling was attempted. Figure 7 shows the blood picture. During the initial period of the bacteremia, there was a leucopenia. The white blood cell count was 5,000 with only 30 per cent mature granulocytes and 50 per cent immature cells. Just prior to cooling, the white count increased and continued to be elevated during hypothermia. Mature polymorphonuclear leucocytes appeared in the high ratio normally expected in severe sepsis. The alterations in hematocrit coincided with the whole blood deficits, and returned to normal with blood replacement.

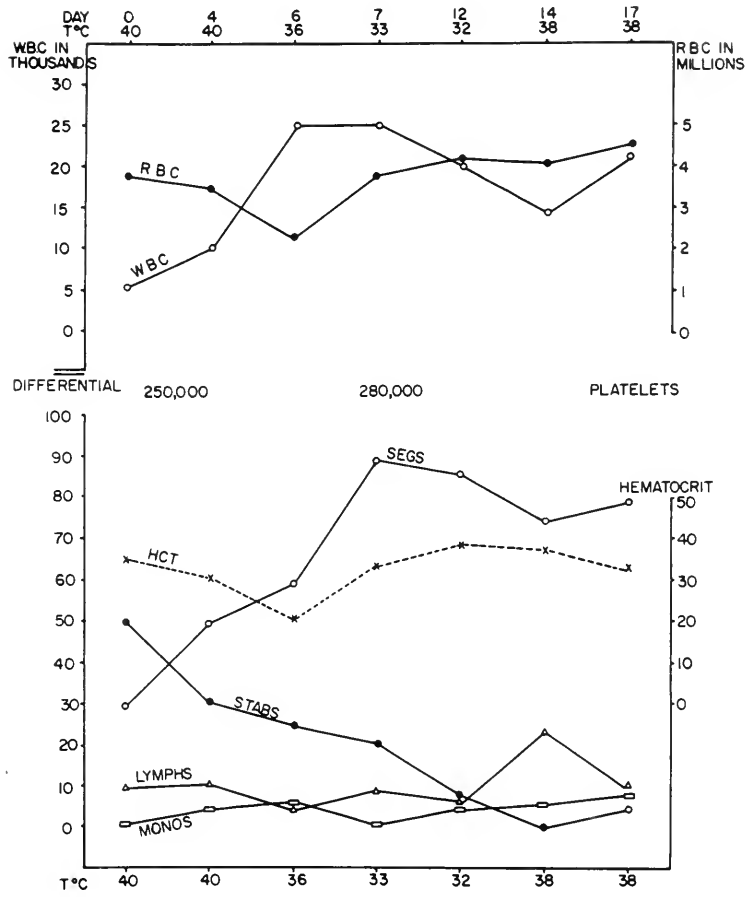


Figure 7. Blood element changes in a case of bacteroides septic shock with hypothermia.

## Discussion of Clinical Observations

Hypothermia proved to be a valuable adjunct in this group of seriously ill patients. Its effect appeared to be primarily metabolic and permitted an increase in cellular tolerance to hypoxia. This "slowing down" allowed time in which to pursue treatment directed specifically against the micro-organism. It is of special interest that death occurred even while bacterio-sensitive antibiotics were being poured in. In part, the failure of antibiosis may be related to

reduced perfusion, the failure of the needed carrier mechanism. The hypoglycemia observed during hypothermia is not in keeping with the usual observations. Hyperglycemia is fairly characteristic in normal animals cooled from 31° C to 25° C (Bickford and Mottram, 1960; Blair, unpublished data) and in humans at 30° C (Henneman et al., 1958). The elevated blood sugar has been attributed to reduced metabolism and impaired absorption (Henneman et al., 1958; Wynn, 1954). Endotoxins have been demonstrated to produce hypoglycemia after an initial hyperglycemia (Berry et al., 1959). Hypoglycemia was not observed in only those patients under hypothermia in this study. One likely explanation may be that shivering caused this, but since all survivors shivered at one time or another, other causes must have existed.

While there is little doubt of the efficacious role of hypothermia in bacteremic shock, the evidence thereto is essentially imperical. The continued administration of antibiotics, corticosteroids, and other drugs may well have obscured the precise effect of hypothermia itself. In order to assess the effect of cooling per se, an experimental study was undertaken.

## EXPERIMENTAL STUDY

### Methods

Randomized chlorolosed mongrel dogs of both sexes weighing between 10-15 kg were induced into septic shock by peritoneal instillation of 1 to 1.5 gms of feces, suspended in saline. Ventilation was unsupported. Three groups of dogs were studied: normal (B), septic shock (S.S.), and septic shock plus hypothermia of 32° C (S.S. + H.). Arterial blood pressure (ABPm) was monitored, as were heart and ventilatory rates (HR and $\dot{V}_R$). Arterial and mixed venous samples and expired air were withdrawn for baseline controls during septic shock and after hypothermia was induced. Oxygen content was determined by Van Slyke-Neil method, while oxygen uptake ($\dot{V}_{O_2}$) was determined by Scholander; and from these cardiac output ($\dot{Q}$) was

| Hours | S.S. + H. | S.S. |
|---|---|---|
| 2 | 0 | 0 |
| 4 | 86 | 13 |
| 6 | 100 | 38 |
| 8 | - | 75 |
| 10 | - | 88 |
| 14+ | - | 100 |

Figure 8. Per cent mortality rates in experimental gram negative septic shock with (S.S.) and without hypothermia (S.S. + H.).

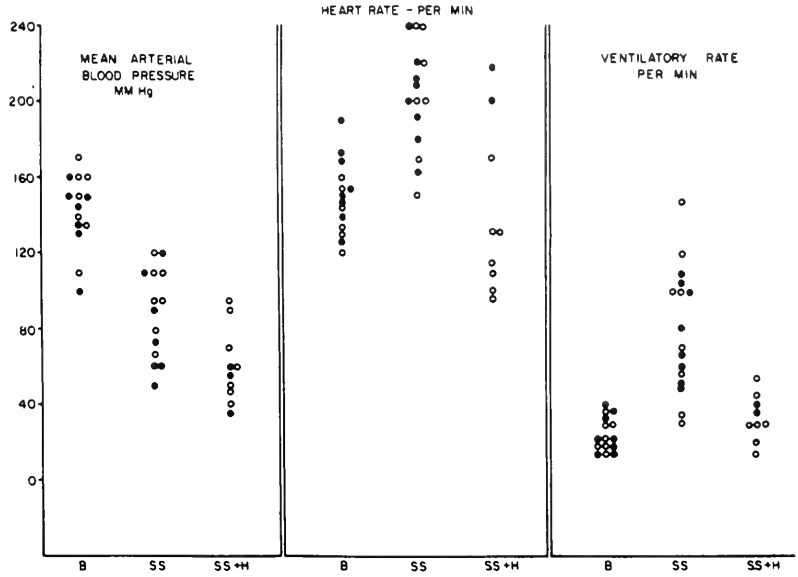


Figure 9. Hemodynamic and ventilatory rate changes in septic shock (closed circles) and septic shock plus hypothermia (open circles).

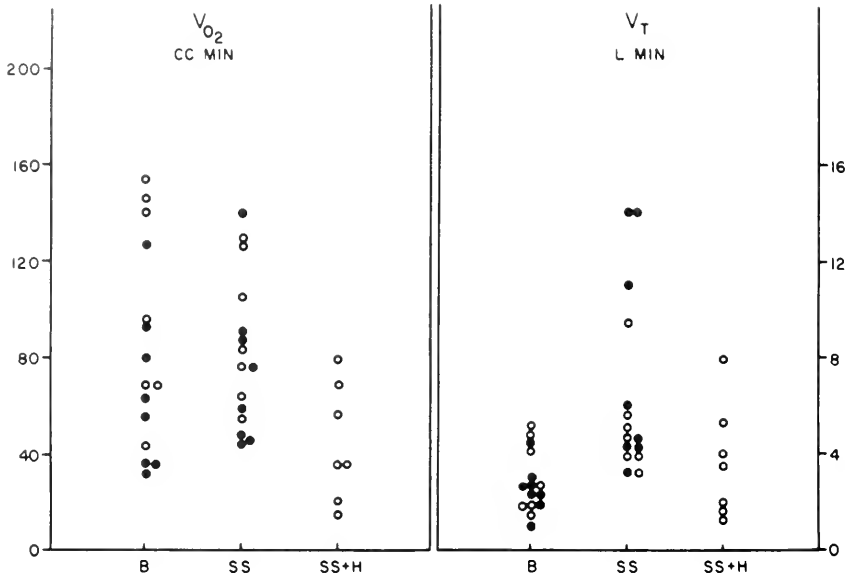


Figure 10. Ventilation in septic shock (closed circles) and septic shock with hypothermia (open circles). $\dot{V}_{O_2}$ = oxygen consumption; $\dot{V}_T$ = minute ventilation. During septic shock, an increased degree of breathing was required to yield a given amount of oxygen. Hypothermia significantly allayed this undue ventilatory work by reducing oxygen requirement ($MRO_2$).

computed. Cultures of the blood stream were done 0, 15, 30, and 60 minutes after instillation of feces. White counts and differentials were done also prior to and after septic shock in both groups. It is emphasized the animals received no fluids or treatment other than hypothermia in the S.S. + H. group, except for replacement of blood removed for analysis.

## Results of Experimental Study

Mortality. Figure 8 shows the mortality rate in both groups. Eighty-six per cent of the animals died in septic shock in four hours, and all died within six hours after fecal instillation. In the hypothermic group 13 per cent died by the four-hour period, 88 per cent by 10 hours, and all by around fourteen hours. Hypothermia did not save any of the animals, but it did prolong survival

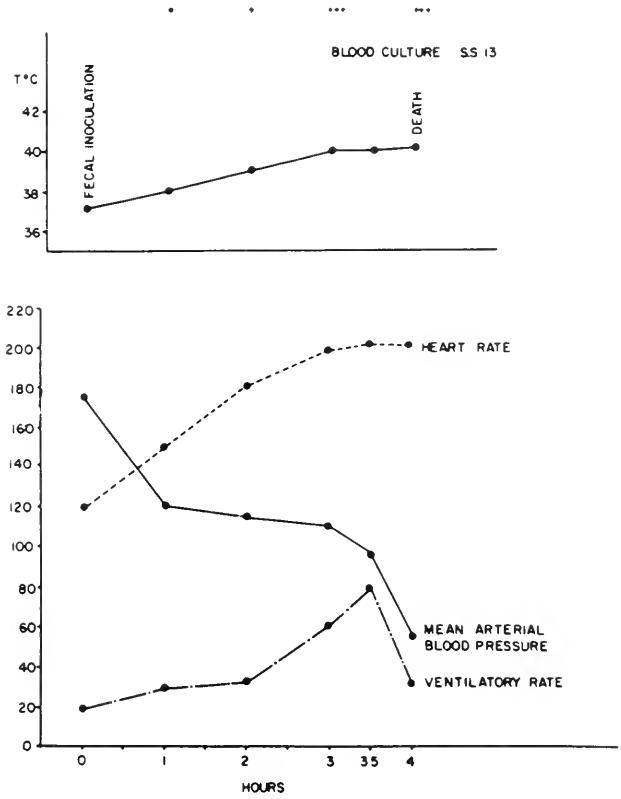


Figure 11. Example of a septic shock study. Concomittant with massive bacterial invasion of the blood stream, the temperature rose, arterial blood pressure dropped, ventilation and heart rates accelerated.

significantly.

Physiologic changes. Figure 9 demonstrates alterations in ABPm, HR, and $\dot{V}_R$. Septic shock was characterized by significant fall in ABPm, by tachycardia, and by hyperpnea. Hypothermia resulted in a further fall in ABPm, and a slowing of HR and $\dot{V}_R$ to or below pre-septic shock values. Non-cooled dogs prior to death underwent serious reduction in the compensating hyperventilation. $\dot{V}_{O_2}$ and $\dot{V}_T$ (minute ventilation) values are shown in Figure 10. $\dot{V}_{O_2}$ in septic shock remained essentially similar to pre-infection, with an increased $\dot{V}_T$. Upon cooling, $\dot{V}_{O_2}$ and $\dot{V}_T$ declined appreciably.

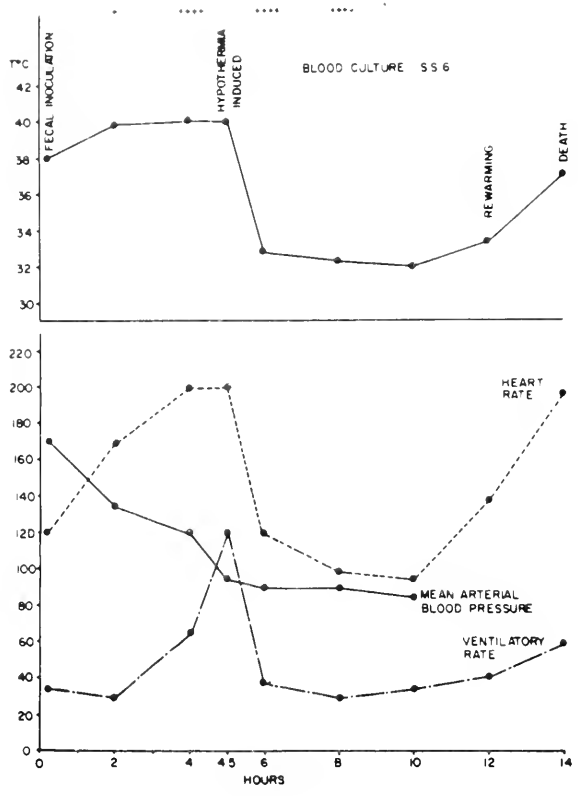


Figure 12. Example of a septic shock and hypothermia study. Note again onset of shock shortly after flooding of blood stream. This likely represents period of failure of clearance mechanisms of the blood stream. Under hypothermia the arterial blood pressure changed little, but ventilation and heart rates slowed considerably. Upon rewarming, heart rate re-accelerated and ventilation decreased, but not to previous high pre-hypothermic level.

Examples of the course of events in an animal from each group are illustrated in Figures 11 and 12. Hypotension, tachycardia, and hyperpnea developed at about the same time the blood stream was overwhelmed with bacteria. The hypothermic dog lived for 14 hours, and then succumbed after it had been rewarmed. Positive blood cultures persisted during the hypothermic period. Effects on A-$\dot{V}_{O_2}$ appear in Figure 13. There was a marked widening (three times normal) which indicated increased extraction of oxygen. At the time of death the A-$\dot{V}_{O_2}$ widened further, while in the cooled septic dogs there

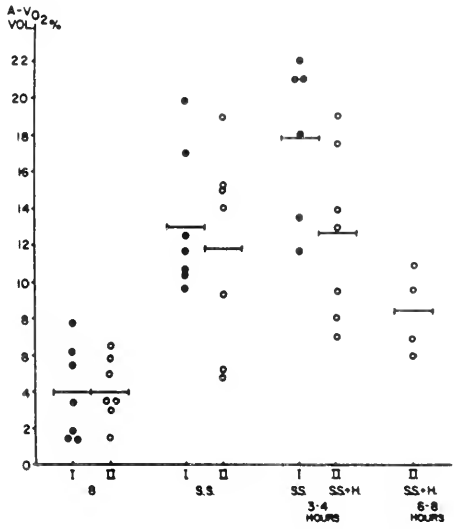


Figure 13. A-$\dot{V}_{O_2}$ differences in septic shock (closed circles) and septic shock with hypothermia (open circles). Baseline (B) values are similar in both groups. During septic shock (S.S.), A-$\dot{V}_{O_2}$ rose with further rise later in the S.S. group. Under hypothermia (S.S. + H.), however, there was significant improvement in A-$\dot{V}_{O_2}$.

| Organism | Per cent |
|---|---|
| E. coli | 62 |
| Streptococcus | 46 |
| Psuedomonas | 23 |
| Aerobacter | 15 |
| Al. faecalis | 8 |

Figure 14. Incidence of predominant micro-organisms cultured from the blood stream.

| Time | Minutes | | Hours | | | |
|---|---|---|---|---|---|---|
| | 15 | 30 | 1 | 2 | 3 | 4 |
| Per cent initial | 30 | 45 | 25 | - | - | - |
| Per cent TNTC | - | - | - | 50 | 37 | 13 |

Figure 15. Percentage rate of appearance time of micro-organisms in blood stream.

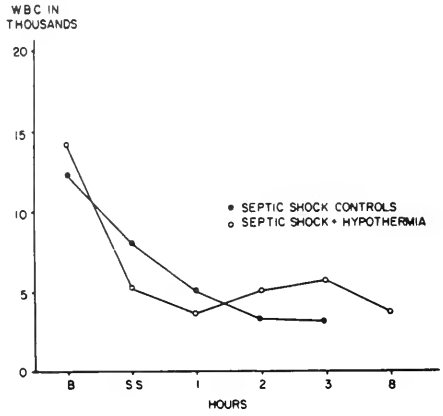


Figure 16. White blood count in septic shock. A marked leucopenia developed with stabilization and some improvement under hypothermia (32° C). Just before death in the hypothermic group, there was a further fall in white blood cells.

was a reduction toward, but not to, normal.

Bacteriology. The most common organism cultured from the blood stream was Escherichia coli (Fig. 14). Cultures included also Streptococcus, Pseudomonas, and Aerobacter. Combinations of E. coli with these were frequent. Bacteria were detected in the blood stream as early as 15 minutes after inoculation (Fig. 15). The blood stream was overwhelmed within two hours, which was about the same time severe shock developed. Cultures remained positive during the hypothermic period.

Leucocytic response. A severe leucopenia developed in both groups, reaching its most advanced state at the time of death (Fig. 16). Induction of hypothermia stabilized the level of white blood cells. There was also a tendency for some increase in count, but

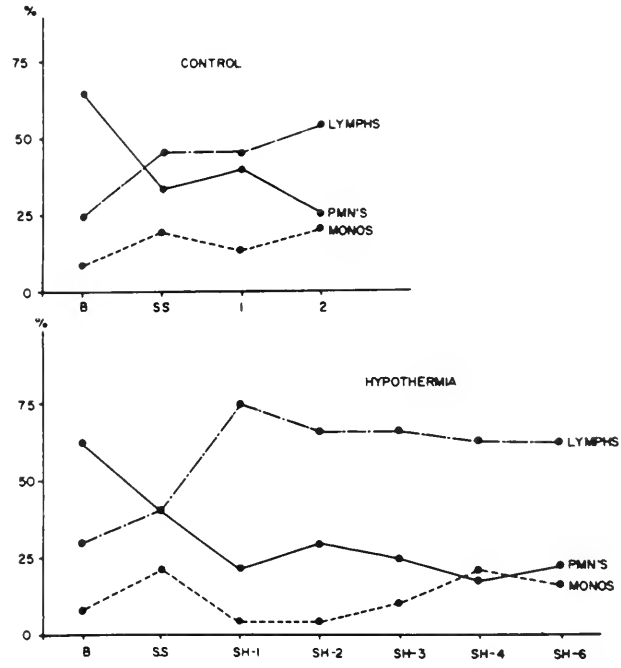


Figure 17. Differential counts demonstrate the leucopenia was due primarily to reduced granulocytes.

the series is too small to attach significance to this. There was a further drop just before death, as occurred in the non-cooled group. The leucopenia was due primarily to disappearance of granulocytes (Fig. 17). Lymphocytes show a relative rise in count with no apparent alteration in monocytes. Cooling resulted in no further change in the differential; only a temporal extention of the pattern was noted.

## Discussion of Experimental Study

Criticism of experimental preparation. Septic shock in the dog is not the same as in the human. Avulsion of the cecum may produce a preparation more similar (Pulaski et al., 1954). In the study performed here the inoculum was homologous. The temporal relationship also was considerably shorter. Better control of bacteriologic

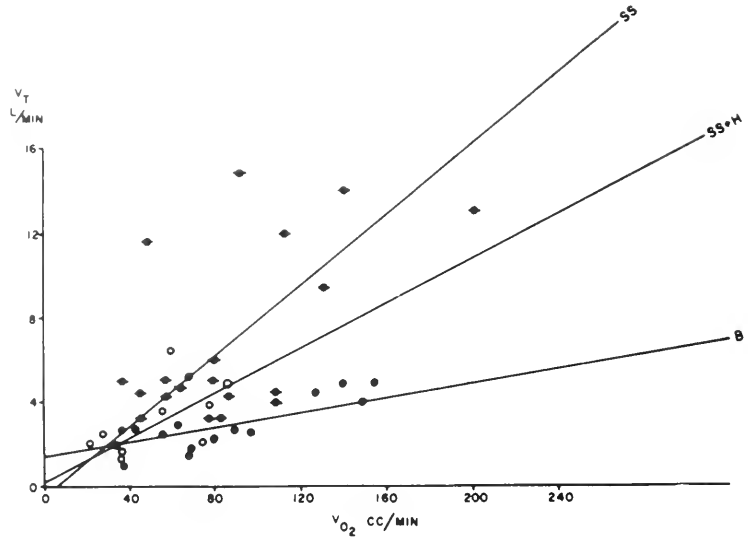


Figure 18. Relation of minute ventilution ($\dot{V}_T$) to oxygen consumption. For details see text.

effects per se may be obtained if a pure strain of E. coli had been used instead of coliform mixtures, which varied with each inoculum.

In this study, interest was centered primarily on the general physiologic and metabolic effects of septic shock alone and as modified by the augmented level of hypothermia. There is less operative trauma with fecal instillation, and the entire study can be completed within 6 to 12 hours. Particularly interesting is the fact that the hemodynamic and hematologic pictures are similar to those caused by endotoxin (Spink, 1960; Gilbert, 1960). The mixed flora is more akin to the clinical insult than pure endotoxin or E. coli. One significant difference between endotoxin and coliform induced changes is that $\dot{V}_{O_2}$ is not decreased as much, whereas $\dot{V}_T$ is greater with coliform sepsis (Maxwell et al., 1960). Increase or no change in $\dot{V}_{O_2}$ and at least an initial increase in $\dot{V}_T$ are characteristic of the clinical syndrome.

Hypothermic effects. Septic shock in these preparations resulted in an increased work load on the cardiopulmonary systems. Response was adequate to a point, but with progressive crippling from the toxemia, compensatory mechanisms broke down. The animal descended

deeper and deeper into hypoxia and finally succumbed from cardiopulmonary failure. The increased ventilatory work required in septic shock is demonstrated in Figure 18. To provide 80 cc of oxygen, normally the dog must breathe 2.8 liters/min. (Curve B). In septic shock (Curve S.S.) an equivalent oxygen availability required 6.2 liters/min. The dog's breathing apparatus had to work 120 per cent harder. When cooled (Curve S.S. + H.), 80 cc of oxygen was obtained with 4.5 liters/ min., reducing work load in septic shock by one-half, but still 60 per cent above normal. Failure of circulation with reduced tissue perfusion has been most often pin-pointed as the main mechanical breakdown leading to death (Spink, 1960; Altmeier and Cole, 1958). These studies are in agreement. Figure 19 demonstrates the relationship between $\dot{V}_{O_2}$ and $\dot{Q}$ in the normal. A and A' represent the approximate range of normal flow required for a given $\dot{V}_{O_2}$. In Figure 20 the effects of septic shock with and without hypothermia are illustrated. Normally, to maintain adequate tissue oxygenation, 100 cc of oxygen is delivered by a minimal flow of 1.1 liters/ min. In septic shock, the equivalent oxygen is supplied by a markedly reduced flow of 0.4 liters/min., a flow deficit of 65 per cent. Induction of hypothermia returned the relationship toward normal. This was achieved primarily by reducing $MRO_2$ with likely little alteration in perfusion. This represents the single most important benefit of hypothermia in this condition. Metabolic environment was brought to a level more commensurate with the sharply compromised capabilities of the physiologic apparatus. The pathophysiologic picture of death in the hypothermic dogs was similar to that in the non-cooled group. The difference is primarily a temporal one. The shock state is due primarily to reduced cardiac output, as is also reported to occur in the human (Gilbert et al., 1955) and in experimental endotoxemia (Maxwell et al., 1960). This is attributed to reduced venous return, secondary to hypovolemia and to pooling (Gilbert, 1960; Ebert and Abernathy, 1961), with the latter occurring primarily in the liver and the splanchnic beds.

The physiologic picture of septic shock in the human and in the dog are similar. Hypothermic effects, however, differ principally in failure to raise ABP in the dog. This may be due to anesthesia or to species difference response. The criteria of septic shock in

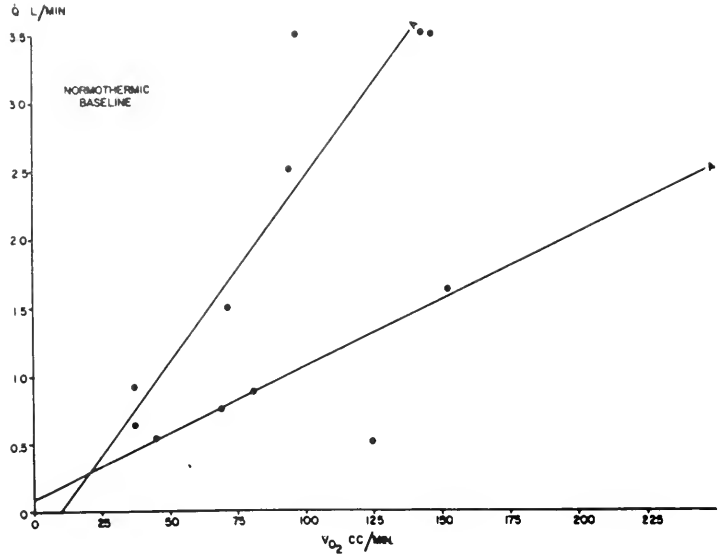


Figure 19. Relation of blood flow ($\dot{Q}$) to oxygen consumption ($\dot{V}_{O_2}$). Curves indicate normal range of flow for a given amount of oxygen.

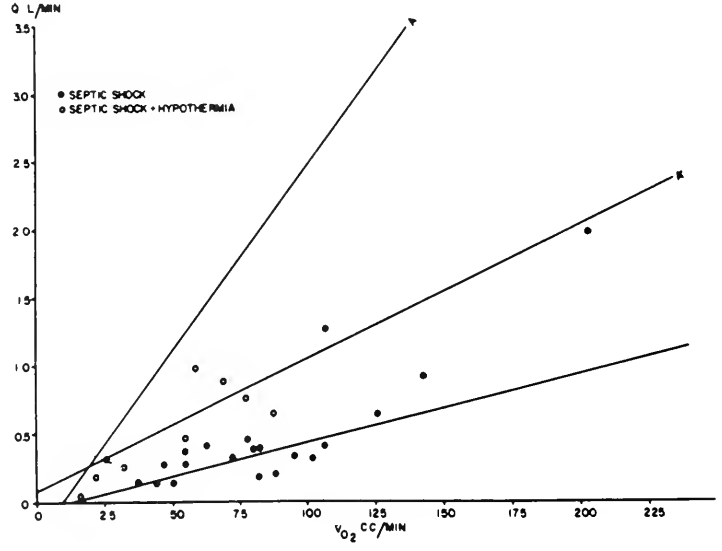


Figure 20. Curves A-A' are reproduced from Figure 11. In septic shock the curve falls toward the abscissa demonstrating reduced flow (closed circles). Under hypothermia the $\dot{Q}$-$\dot{V}_{O_2}$ relationship is improved.

the dog are hypotension, hyperpnea, tachycardia, and increased A-$\dot{V}_{O_2}$. In the human they are hypotension, tachycardia, and a reduced and irregular ventilation, and widened A-$\dot{V}_{O_2}$.

Leucopenia. The leucopenia is reported due to endotoxic effect and the hyperpyrexia due to pyrogens from granulocytes. The mechanism is obscure, but may be in part caused by depression of bone marrow activity. The breakdown of host resistance (Zweitach et al., 1957) lies in part to depression of the reticuloendothelial system. Direct endotoxin effect or reduced perfusion or both may contribute to the RES breakdown. Granulocytes were affected primarily in this study. However, counts were made on whole blood, and the increased hematocrit in this type of shock may introduce an error in the cell counts.

The persistence of positive blood cultures under hypothermia indicated no significant effect on the bacteria at the temperature used. Lower temperatures are required for possible influence on growth (Balch et al., 1955).

Hypothermia presumably does not alter the blood's ability to clear bacteria (Fedor et al., 1956; Frank et al., 1956). Previous experiences on the effect of hypothermia in experimental infections have produced conflicting reports. Increased mortality rate was observed in rabbits with pneumococcal infection after cooling to 31° C (Sanders et al., 1957). A similar study at 21° C yielded an increased survival rate (Wotykins et al., 1958). Death rates in rats from gram negative septicemia were improved with cooling to 25° C (Balch et al., 1955). The variations in results may well be due to species differences, depth of cooling and virulence of the bacteria used.

## SUMMARY

Bacteremic shock is usually of gram negative coliform origin. The mortality persists at 65 to 70 per cent despite appropriate

antibiosis and supportive therapy. The underlying physiologic dysfunction appears to be hypoxia secondary to a tissue perfusion deficit. The rationale of hypothermia lies in the reduction of $MRO_2$ in order to place tissue needs at a level more commensurate with the reduced blood flow. The action of hypothermia is non-specific. Its effect lies in the modification of the host metabolic environment. The augmented level of $32^{\circ}$ C has proved effective and safe and likely does not alter bacterial growth.

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## DISCUSSION

BERRY: I would like to take just a few minutes to tell about some very preliminary findings we have at Bryn Mawr, perhaps relevant to the problem that Dr. Blair emphasized so clearly; the problem of cell anoxia in the patient with septic shock. I want to preface my remarks by recalling my early years as a scientist, the era of vitamin research. Animals were made vitamin deficient and had all sorts of symptoms. It seemed hopeless to find what the vitamin was doing metabolically, because the symptoms were so diffuse, and this is very much the problem with endotoxin.

Now, the metabolic effects of endotoxin are so diffuse and so wide-spread in an animal, it may be impossible to ever pinpoint the specific enzyme that might be involved. I'm not sure that I have found the enzyme, but I think there is one enzyme inhibited by endotoxins, and this enzyme is important to the whole well-being of the animal, and may be a valuable clue. The enzyme is tryptophan pyrrolase. It converts tryptophan oxidatively into knyurenine which, in turn, is changed into nicotinamide, which is incorporated in the pyridine nucleotides DPN and TPN. These are the primary hydrogen acceptors in oxidative metabolism, and they are, therefore, directly involved in most of the energy release of the mammalian organism. The reason that I started looking at this enzyme is because the glycocorticoids of the adrenal cortex which are the most effective, if not the only effective compounds capable of protecting animals against endotoxin, cause a prompt and large increase in tryptophan pyrrolase in the livers of experimental animals. One finds, also, that tryptophan will cause an increase in tryptophan pyrrolase, probably as a kind of an adaptive enzyme response. If the enzyme, tryptophan pyrrolase, really plays an important role in the protection of an animal against endotoxin, then tryptophan should be about as effective as cortisone in protecting against endotoxin. This was not found with tryptophan, much to our surprise, but it was observed with niacin at a level of 20 $\mu$M, while DPN, at 0.3 $\mu$M, was also active. This is what one would predict if the

protection is dependent upon the pyridine nucleotides. An encouraging part of this study is the fact that an analogue of niacin, 3-acetyl pyradine, produces in mice some of the symptoms of endointoxication. The ruffled fur, the apparent drop in blood pressure, the adrenal insufficiency, etc. are obtained with this nicotinic acid analogue. If one waits a matter of only four hours after giving endotoxin and then attempts to protect with any of these compounds, none is effective. I don't know why. But at least there is hope that we may have one level of explanation for the metabolic events that produce cell anoxia, Dr. Blair. We need to do many more experiments. I hope what I report now stands up on repetition.

BLAIR: With regard to Dr. Berry's very exciting observations, there is no question but that before this matter of endotoxic shock, if that is really what it is, can be handled adequately by physicians, you have to know a great deal more about the basic, fundamental changes, and this is certainly an approach to that. I personally would be most interested in this in relation to oxygen tensions, because there has to be some relationship as to whether it is the endotoxin itself that produces this deficiency, or is the hypoxia secondary. In other words, is it simply a matter of lack of oxygen?

Just in line with this, we at the University of Maryland have become interested in the use of hyperbaric oxygen and treatment of varied and sundry illnesses. This is going to be the latest gimmick on the surgical scene now, and I have taken two of our dogs in septic shock, thrown them into a modified chamber at three atmospheres of whole oxygen, and brought out two dead animals, so I don't think that this particular approach is necessary. Maybe we gave them too much oxygen.

With regard to the matter of the hypothermia and the pseudomonas infections, on the surface, the matter seems to be an incongruity from the standpoint of recommendations for cooling a patient. On the one hand, it seemed that hypothermia is probably a bad thing to use, and certainly one gets in to very serious trouble if he allows his patient either spontaneously or under deliberate cooling conditions, to drop down to too deep a level.

In general, we observe patients who are about to go in shock to show a slight fall in temperature. The extreme hyperpyrexia does change. He still has a high fever, but there is usually a drop of $1^{O}$ C on the average. Apparently the hyperpyrexia is related to the problem of the protective coverings of the skin. It's pretty important, apparently. Hypothermia in experimentally burned animals is quite a difficult problem to cope with. There have been several attempts to use hypothermia in burned animals, and none of them have been successful. Now, whether this burn septicemia itself is a separate entity or somewhat different than those from germs that come out of the colon, I don't know, but certainly there may well be some very significant differences in the toxins of these two circumstances.

Concerning cold therapy, there is no such thing as cold therapy at all. Cold doesn't treat anything. Cold modifies metabolic environment. Sometimes it is good, sometimes it is bad. The point of the matter is that use of hypothermia does not dictate the ultimate survival of the patient, or certainly of the experimental animals. It is only adjunct, and it is a crutch. Heaven knows we certainly need crutches in treating very sick people, and hypothermia has been somewhat useful in that respect.

# SUMMARY OF SYMPOSIUM

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Years ago, the great naturalist Agassiz counselled, "Go to Nature. Take the facts into your own hands. Look and see for yourself." During the past three days, we have followed this sage advice. But as we have looked, perhaps we have not seen with complete clarity, for the problems are complex. Yet those responsible for this symposium have given us a widely arranged spectrum of subjects for discussion which have afforded us the opportunity to 1) "go to Nature" and look at the effects of cold on the incidence of naturally acquired infections in man; 2) "take the facts in (our) own hands" and see for ourselves the effects of hypothermy on experimental infection; and finally, 3) hear reports on the search for mechanisms by which host-parasite interactions are affected by hypothermy.

In examining the data on the effect of cold on naturally acquired infections in man, we are confronted by complexities, as was clearly pointed out by Dr. Berry, Dr. McClaughry, Dr. Babbott, and Sir Christopher Andrewes. In determining whether or not lower ambient temperatures affect host-parasite relations, it has been shown that a number of factors must be considered, among which may be listed the following: 1) the temperature gradient between normal body temperature and the environment; 2) moisture or wind which increase the transfer of body heat to the environment with a local and general cooling of the body; 3) non-uniform cooling on parts of the body which may have peculiar physiological effects, as demonstrated by Mudd et al. (1921); 4) since we know from Pfluger's Law that the physiological response is related more directly to the rate of change of the stimulus than to the strength of the stimulus, rate of change of body temperature is significant; 5) physiological conditions of the host affecting heat transfer from the skin as peripheral dilation (alcohol) or the effects of local or general cooling of the body as cardio vascular diseases, endocrine disturbances, and so forth; 6) previous acclimatization of the subject to cold, whatever this means

physiologically; and 7) pathogenic microbial flora of the host or his environment at the time of exposure to low temperature.

To suppliment all this, the question might be asked, does an excessive heat transfer from the host to his environment affect host resistance to infectious diseases? As a starting point, we should ask ourselves what has been decided as to the effect of low ambient temperatures on the resistance of man to infectious diseases. As Dr. Berry recalled, many of his elders as well as his country doctor all warned against wet feet (and heat loss). At that time, these people, being fine Texans, believed that exposure to cold did predispose to upper and lower respiratory tract infections. Of course proof of such widespread beliefs is hard to come by, as Dr. McClaughry and Sir Christopher have stated. Goldstein (1951) has claimed that extreme cold is a "potent stress factor in bringing about the common cold". Troisi et al. (1953) found a higher incidence of upper respiratory infections in 66 men working in cold rooms of meat preservation plants. Andrewes has shown us that colds are more prevalent in temperate zones in winter than in summer, and as we know, the rate of change of heat loss can be quite marked in these zones. The chances for irregular cooling of body surfaces may be significant. But I think we would all agree that a definitive answer has not yet been derived.

It would appear that we need more epidemiological evidence on the effect of increased heat transfer from the host and on susceptibility to infection. Experiments in the future should attempt to correlate not cold as such, but rather, the various factors associated with general or local loss of body heat with the incidence of infection. And what are these various factors? If only some knowledge as to what measure to use other than rectal temperature or ambient temperature were available. Perhaps then we would have a more direct measure of what exposure to cold does to the host, and by this many of the variables which now confront us might be reduced considerably.

Turning now to another aspect of the symposium, we might recall the reports on experiments with animals in which host resistance was altered by exposure to low ambient temperatures. Conclusive data was presented by Dr. Previte and Dr. Miraglia which showed

that host resistance to selected strains and doses of salmonella and staphylococci was decreased by a low environmental temperature. Dr. Metcalf and Dr. Walker demonstrated that in chilled animals there is a lowered resistance to influenza and Coxsackie B-1 viruses respectively. And Dr. Marcus has shown us that chilled mice which were not acclimatized are more susceptible to Coxsackie B-5 virus than are normal animals. On the other hand, Dr. Sulkin has told us that there is less rabies or encephalitis virus produced in bats at $-2^{\circ}$ C than at higher temperatures. These findings clearly indicate the complex nature of the problems we face. It is indeed logical to expect less virus production in cells whose metabolism is lowered by cold; but pathogen replication is only one phase of disease production, although it is an important one.

Another aspect of these experiments conducted with animals is that it became clear that in challenging them while kept at normal or low ambient temperatures, one must define certain experimental procedures quite carefully. These include such aspects as ambient temperature, rectal temperature, cage type, bedding (or lack of bedding), acclimatization (which should be defined by physiological measurements, if possible), virulence and dosage of pathogen, and site of inoculation (S. C. or I. P. being the major methods used).

Dr. Walker's report on virus replication showed that the strain of Coxsackie used was replicated in the pancreas of animals kept at normal temperatures, but was not replicated in other tissues. Infections of the pancreas are rare, yet this strain of Coxsackie selects this organ as its site of operations in the normal animal. When the animals were chilled in Dr. Walker's experiments, the virus became replicated in many tissues.

Dr. Berry cited Shephard's success in producing growth of M. leprae in mice injected in the foot pad, and Shephard has surmised that success was based on the lower temperatures of foot pad tissue. We all know that T. pallida will not infect rabbit testicles unless the ambient temperature is lower than $59.5^{\circ}$ C. Also, elevated body temperatures have been used in man to treat syphilis. One wonders if these effects are related to the metabolism of the pathogen or host defense mechanisms. It is of some interest to observe that certain pathogens such as the trypanosomes and leptospira grow much

better in vitro at 30° C than at 37° C, yet in vivo they do very well at 37° C.

If we accept the evidence currently available, we must conclude that cold does lower animal host resistance to many infectious agents. The question with which we must concern ourselves is, "How?"

Host resistance to infectious agents involves anatomical, physiological, biochemical, and immunological factors, and most of these have been touched upon by the various participants here.

Anatomical. Any break in the skin resulting from frost bite or cold sensitization obviously increases the chances of infection. Some of the most serious infections of man result from such losses of mechanical protection of the skin, as can be seen in the infection which can complicate burns. Much more subtle anatomical factors such as the blood supply of the skin, the nasal mucosa, and the architecture of the upper respiratory tract may be concerned with this problem.

Physiological. The physiological changes in the host resulting from sudden or prolonged exposure to cold are even more subtle. Changes of this type have been called to our attention by Dr. Blair and Dr. Miya. The latter has shown that there is an increased resistance to bacterial endotoxins in the cooled animal, while Dr. Blair and Col. Moncrief have described their use of hypothermy to treat bacterial shock in man. Probably the increased resistance of chilled animals and man to endotoxin is based on some physiological mechanism which has not yet been defined. As Sir Christopher pointed out, disease production and mechanisms related to it are important aspects of this story. And the report of Washburn (1962) on the changes in blood circulation in frost bitten skin is pertinent to the lowering of skin resistance by cold.

My own experience in this area relates to the mucus secretions in the upper respiratory tract. It is recognized that sterile hog gastric mucin (Nungester et al., 1936; Olitzki, 1948) and human respiratory tract mucin (Nungester et al., 1951) lowers host resistance to bacterial infections. Experimental pneumonia can be produced readily by injecting a suspension of bacteria and mucin deep into the res-

piratory tract of dogs or rats (Nungester and Jourdonais, 1936). Also, pneumococci and mucin placed in the nose of rats will produce experimental pneumonia under certain conditions which alter the normal physiological defense mechanisms (Nungester and Klepser, 1938). But do such findings have any relation to cold and respiratory tract infections?

Mudd et al. (1921) have shown that an uneven cooling of skin surfaces causes vascular changes in the nose. It is common for nasal secretions to flow out of the anterior nares when one comes into contact with cold air. Either the ciliary mechanisms fail or there is a marked increase in secretions. It seems to me that the latter is more correct, for it is based on a sudden change in blood supply to the mucus secreting glands. This hypothesis is based on the early findings of Mudd and his colleagues. A marked increase in respiratory tract secretions based on physiological stimulation (cold air) or infections will overburden the cilia and cause the mucus secretions to accumulate in the upper respiratory tract. Such an oversupply may drain out the anterior nares, be swallowed, or might possibly be aspirated. Is it possible, then, that marked cooling of the body may increase the chances for aspiration of infected mucus secretions from the upper respiratory tract?

We have some evidence from our laboratory (Nungester and Klepser, 1938) which showed that the reflex control of the glottis is decreased by body chilling. Mechanical stimulation of the glottis area with a small wire in lightly anesthetized rats resulted in closing of the glottis in all but 18 per cent of 754 stimulations. With rats chilled for ten minutes in ice water, the glottis did not close in 46 per cent of 552 stimulations. In 21 other rats, none of them aspirated India ink colored mucin placed in the nose, while 55 per cent of 20 rats previously chilled aspirated the material into the lungs. Pneumococcus pneumonia developed in 13 per cent of 46 normal rats inoculated internasally with mucin and pneumococcus. In another group of 24 rats, chilled in ice water and similarly injected, 42 per cent developed pneumonia. These results may or may not be related to severe chilling and pneumonia in man.

Dr. Tunevall has told us that absorption of tetanus toxin is delayed in the hypothermic animal. This directs our attention to the physi-

ology of the lymphatics and the peripheral circulation. Possibly the increased resistance of the hypothermic animal to bacterial endotoxin reported by Dr. Miya and the clinical findings of Dr. Blair are directly related to the findings of Dr. Tunevall. We might call attention to the findings of Klepinger et al. (1959) that of 58 drugs tested, all but strychnine, chlorpromazine, and promazine were more active in animals kept at 36° C than at 3° C or at 26° C.

Biochemical changes. Does exposure to cold produce any measurable effects on the biochemistry of the host? Dr. Trapani has mentioned the increased thyroid activity of the hypothermic animal. This is significant. Dr. Campbell has called our attention to the absence of the beta anomaly in the descending electrophoretic pattern in the serum of a man living in an arctic or subarctic climate. He also mentioned the significance of the brown fat found in the hibernating squirrel. Dr. Sulkin was particularly interested in this brown fat in the hibernating bat. The question arises as to what role this peculiar tissue with its high lipid content plays in hibernation or resistance to the effects of cold.

Another interesting biochemical find has come to light in the work of Monier and Weiss (1952). A sharp increase in the excretion of ascorbic acid (53 per cent) and dehydroascorbic acid (186 per cent) was noted in hypothermic animals over normal animals. Since ascorbic acid is found in large quantities if phagocytic cells and must be present to a certain level in the phagocytes are to operate properly, such losses of this essential vitamin must be compensated for, or host resistance may be lowered through loss of ascorbic acid.

Other biochemical changes in hypothermic animals have been noted which may or may not be related to changes in host resistance. For example, Ershoff (1951; 1952) noted a decreased resistance of pyridoxine on riboflavin deficient rats which had been chilled. Ultimately, we will better understand host-parasite relations when adequate knowledge is available to explain such phenomena on a biochemical basis. Dr. Metcalf's report on the effects of temperature on the neuraminidase of the influenza virus in embryonated eggs and in the mouse lung represents such a biochemical approach to the problem.

## SUMMARY

Immunology. The effect of cold on circulating antibody was discussed by Dr. Campbell. Two factors which he pointed out that should be kept in mind were 1) the effects of cold on antibody production and antigen breakdown, and 2) the effects of cold on antibody removal from the circulating blood.

In general, the participants reported a decrease in circulating antibody in chilled animals. However, Dr. Northey found little difference.

Cold and microbial flora of host. The effect of maintaining mice at $22^{\circ}$ C to $23^{\circ}$ C on the invasion of the blood stream and peritoneal cavity, as reported by Dr. Tunevall, emphasizes how little we know as to the permeability or impermeability of the gut to the passage of macromolecules or microorganisms. This permeability might not be of significance except that as Dr. Tunevall reports, clearance of bacteria from the blood stream is also impaired in the hypothermic animal. A basic explanation of both findings is in order.

Another mechanism by which cold may alter host microbial flora is suggested by Dr. Schmidt's report on the intestinal flora of squirrels as affected by hibernation. He found a sharp increase in psychrophilic bacteria. Such a change in intestinal flora, induced by hypothermia, could be associated with changes in available vitamin K produced by the normal intestinal flora and more significantly, with an increase or decrease of antimicrobial agents produced by intestinal bacteria.

## SUMMARY

In conclusion, it is safe to say that the final blue print showing the anatomical physiological biochemical and immunological mechanisms by which exposure to cold alters host resistance to infection will not be simple. But it will be a workable paper and will suggest practical ways of increasing host resistance.

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